Trained sensitization and tolerance development in microglial cells –

Role of phosphoinositide 3-kinase γ

Dissertation

zur Erlangung des akademischen Grades

Doktor der Philosophie (Ph.D.)

vorgelegt dem Rat der Medizinischen Fakultät

der Friedrich-Schiller-Universität Jena

von Magister der Pharmazie (Mag. pharm.)

Trim Lajqi

geboren am 04.01.1990 in Peja, Kosovo

Gutachter:

1. Prof. Dr. Reinhard Bauer (Friedrich-Schiller Universität Jena, Jena)

2. Prof. Dr. Reinhard Wetzker (Friedrich-Schiller Universität Jena, Jena)

3. Prof. Dr. Peter Brust (Helmholtz-Zentrum Dresden-Rossendorf, Leipzig)

Tag der öffentlichen Verteidigung: 23.04.2019

Table of contents

List of figures

List of tables

List of abbreviations

AMPK	Adenosine monophosphate-activated protein kinase
ANOVA	Analysis of variance
Arg-1	Arginase-1
ATP	Adenosine triphosphate
BCG	Bacillus Calmette-Guérin
BDNF	Brain-derived neurotrophic factor
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CD14	Cluster of differentiation 14
cDNA	Complementary DNA
CLRs	C-type lectin receptors
CNS	Central nervous system
DAMPs	Damage-associated molecular patterns
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's modified eagle medium
dsRNA	Double-stranded RNA
ECAR	Extracellular acidification rate
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FCS	Fetal calf serum
FITC	Fluorescein-isothiocyanate
GPCRs	G protein-coupled receptors
H_2DCFDA	2',7'-dichlordihydrofluorescein-diacetat

H3K27Ac	Acetylation of lysine 27 residue on histone 3
H3K4me3	Trimethylation of lysine 4 residue on histone 3
H3K9me3	Trimethylation of lysine 9 residue on histone 3
HIF-1α	Hypoxia-inducible factor 1-alpha
HRP	Horseradish peroxidase
IL-10	Interleukin 10
IL-1β	Interleukin 1-beta
IL-4	Interleukin 4
IL-6	Interleukin 6
iNOS	Inducible nitric oxide synthase
KD	Kinase-dead
KO	Knock-out
LPS	Lipopolysaccharide
MD-2	Myeloid differentiation factor 2
MMPs	Matrix metalloproteinases
mTOR	Mechanistic target of rapamycin
MyD88	Myeloid differentiation factor 88
NFκB	Nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
NO	Nitric oxide
OCR	Oxygen consumption rate
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDE	Phosphodiesterase
PFKFB3	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3

PI3K	Phosphoinositide 3-kinase
PI3Kγ	Phosphoinositide 3-kinase gamma
PIP_2	Phosphatidylinositol-4,5-bisphosphate
PIP_3	Phosphatidylinositol-3,4,5-trisphosphate
PKA	Protein kinase A
PKB (Akt)	Protein kinase B
PRRs	Pattern-recognition receptors
PTEN	Phosphatase and tensin homolog
ROS	Reactive oxygen species
RTKs	Receptor tyrosine kinases
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
TBS	Tris buffered saline
TBS-T	TBS-Tween
TGF-β	Transforming growth factor- beta
TLR4	Toll-like receptor 4
TLRs	Toll-like receptors
TNF-α	Tumor necrosis factor- alpha
TRIF	Toll/IL-1 receptor (TIR) domain-containing adaptor
Vps34	Vacuolar protein sorting 34
WT	Wild type

Summary

Ongoing investigations during the last decade showed that innate immune cells are capable to induce memory-like (adaptive) responses such as trained sensitization and tolerance, after repeated challenge by the same or different stressors (PAMPs). These effects are imposed by epigenetic modifications and followed by metabolic changes of immune cells, resulting in partly long-lasting alterations in their inflammatory response, thus affecting different cellular processes of innate immunity.

Recent investigations identified the pathogen dose, in addition to pathogen molecule, as critical factor for the mediation of trained sensitization - priming by low-dose PAMPs, and tolerance - priming by high-dose PAMPs, suggesting a dose-dependent induction of enduring immune patterns in innate immune cells. These findings characterize different metabolic rearrangements as critical for the induction of two contrasting patterns of immune response: trained sensitization where low amounts of PAMPs provoke resistance mechanisms aiming at pathogen elimination leading among others to increased production of pro-inflammatory cytokines, as well as tolerance induced by high loads of PAMPs shifting from resistance to maintenance and repair mechanisms resulting with a response profile characterized by a dampened pro-inflammatory cytokine release.

Microglial cells as long-living resident immunocompetent cells of the CNS represent macrophage-like cells that survey the brain microenvironment in order to secure homeostatic conditions for typical neural functions. Any pathological event or injury in CNS triggers the activation of microglial cells exhibiting a wide range of phenotypes and functions aiming either to induce maintenance and repair mechanisms or to worsen the severity of the injury in the brain. Furthermore, these events depend on various molecular signatures induced by different stressors, e.g. pathogen conserved structures such as PAMPs as well as damage associated molecular patterns (DAMPs) resulting in distinct response patterns of microglial cells such as cytokine profiles as well as and other immune cell functions (e.g. phagocytosis).

Phosphoinositide 3-kinase γ (PI3Kγ) as a major signaling protein is activated by G protein-coupled receptors (GPCRs), thus triggering the rapid phosphorylation of phosphatidylinositol-

4,5-bisphosphate (PIP_2) into phosphatidylinositol-3,4,5-trisphosphate (PIP_3) resulting in activation of protein kinase B/Akt downstream signaling mechanisms. In addition, studies have shown that PI3Kγ can bind and subsequently induce the activation of other proteins such as phosphodiesterases (PDE), modulating cAMP-related signaling. This scaffold function indicates a lipid kinase-independent activity in addition to its well-characterized enzymatic properties. Previous group data have reported that lipid kinase-dependent and -independent activities of PI3Kγ are key mediators of different microglial functions such as migration, proliferation, phagocytosis, as well regulating the production of ROS and cytokines, representing PI3Kγ as a putative signaling protein important for the induction of trained sensitization and tolerance.

The aim of this study was to investigate the dose-dependent induction of trained sensitization and tolerance by different pathogen conserved structures (LPS, β-glucan) in microglial cells. Furthermore we hypothesized that PI3Kγ regulates different metabolic changes, critical for the development of adaptive responses, thus affecting microglial functions such as phagocytosis.

Our data revealed that microglial cells, when primed by low-dose PAMPs (LPS, β-glucan) and challenged subsequently with the same or different pathogen, respond with increased production of pro-inflammatory cytokines – trained sensitization. In contrast, cells primed by high-dose PAMPs induce a robust tolerance phenotype with reduced pro-inflammatory and increased anti-inflammatory response. The induction of trained sensitization and tolerance was mediated by different metabolic changes (glycolysis and oxidative phosphorylation), affecting the microglial phagocytosis. Furthermore, our study revealed a tight regulation of the cytokine profile, metabolic events and phagocytosis by lipid kinase-dependent and -independent activities of PI3Kγ in microglial cells.

Zusammenfassung

Neuere Untersuchungen des letzten Jahrzehnts zeigten, dass Immunzellen der angeborenen Immunabwehr in der Lage sind, Gedächtnis-ähnliche (adaptive) Reaktionen, d.h. trainierte Sensibilisierung bzw. Toleranz, auszuprägen, nachdem sie mit denselben oder verschiedenen Stressoren (PAMPs) mehrfach konfrontiert wurden. Diese Wirkungen werden durch epigenetische Modifikationen und resultierende metabolische Veränderungen verursacht, die mit der Induktion von Entzündungsreaktionen der Zellen einhergehen und dabei verschiedene zelluläre Prozesse der angeborenen Immunität beeinflussen.

Aktuelle Untersuchungen identifizierten die Stressoren-Dosis zusätzlich zum jeweiligen Stressoren-Molekül als kritischen Faktor für die Vermittlung von trainierter Sensibilisierung (Priming durch niedrig dosierte PAMPs) sowie Toleranz (Priming durch hochdosierte PAMPs), was auf eine dosisabhängige Induktion dauerhafter Muster von Immunantworten der angeborenen Immunabwehr hindeutet. Diese Befunde charakterisierten dabei verschiedene metabolische Umstellungen als kritisch für die Induktion dauerhafter Muster von Immunantworten der angeborenen Immunabwehr: Induktion von trainierter Sensibilisierung, induziert durch geringe Mengen von PAMPs, die Resistenzmechanismen zur Pathogeneliminierung provozieren und dabei zu einer erhöhten Produktion von proinflammatorischen Zytokinen führen; Toleranz, induziert durch hohe PAMPs-Dosen, dass durch Aktivierung von Reparaturmechanismen zu Resistenzverhalten führt und dabei unter anderem eine Dämpfung des proinflammatorischen Profil von Immunzellen zur Folge hat.

Mikrogliazellen als langlebige residente immunkompetente Zellen des ZNS überwachen fortlaufend die Mikroumgebung im Gehirn, um homöostatische Bedingungen für die neuronalen Funktionen zu sichern. Jedes pathologische Ereignis, insbesondere jede Verletzung im ZNS löst die Aktivierung von Mikrogliazellen aus, die ein breite Spektrum von Phänotypen und Funktionen annehmen können, die entscheidend dazu beitragen, Reparaturmechanismen zu induzieren oder daran beteiligt sein können, die Schwere der Verletzung im Gehirn zu verschlimmern. Obgleich die Details zur Regulation der jeweiligen zellulären Reaktionsmuster noch unzureichend verstanden sind, wurde nachgewiesen, dass letzteres durch verschiedene konservierte molekulare Signaturen entscheidend beeinflusst wird. Dies schließt neben

körpereigenen Stressoren (Alarmine), die bei Verletzungen oder Zellschädigungen freigesetzt werden (DAMPs), insbesondere pathogenkonservierte Signaturen, wie PAMPs ein und modifiziert maßgeblich auch das Zytokinprofil aktivierter Mikrogliazellen einschließlich anderer immunrelevanter zellulärer Funktionen (z. B. Phagozytose).

Phosphoinositid-3-Kinase γ (PI3Kγ) als Signalprotein wird durch G-Protein-gekoppelte Rezeptoren (GPCRs) aktiviert, wodurch eine rasche Phosphorylierung von Phosphatidylinositol-4,5-bisphosphat (PIP_2) zu Phosphatidylinositol-3,4,5-trisphosphat (PIP_3) resultiert und eine forcierte Aktivierung von Protein Kinase B / Akt -abhängigen Signaltransduktionsmechanismen verursacht wird. Darüber hinaus haben Studien gezeigt, dass PI3Kγ Proteinkomplexe ausbilden kann, das deren Aktivierung zur Folge hat (wie z.B. Phosphodiesterasen), wodurch u.a. cAMP-abhängige Signalwege modifiziert werden können. Somit weist PI3Kγ neben seinen enzymatischen Eigenschaften auch Lipidkinase-unabhängige Funktionen auf. Frühere Untersuchungen der Arbeitsgruppe wiesen nach, dass Lipidkinase-abhängige und -unabhängige Aktivitäten von PI3Kγ wesentliche Funktionen aktivierter Mikroglia- regulieren (u.a. Migration, Proliferation, Phagozytose sowie die Produktion von ROS und Zytokinen). Somit war es naheliegend zu vermuten, dass PI3Kγ auch ein relevantes Signalprotein für die Regulation von trainierter Sensibilisierung und Toleranz darstellen könnte.

Das Ziel dieser Studie war es zu untersuchen, ob eine dosisabhängige Induktion von trainierten Sensibilisierung und Toleranz durch unterschiedliche pathogene Stressoren (LPS, β-Glucan) bei Mikrogliazellen nachzuweisen ist. Darüber hinaus stellten wir die Hypothese auf, dass PI3Kγ verschiedene metabolische Veränderungen reguliert, die für die Entwicklung adaptiver Antworten von Mikrogliazellen auf repetitive Stressorkontakte von entscheidender Bedeutung sind und dadurch Modifikationen adaptiver Immunantworten, wie Phagozytose-Muster beeinflussen.

Unsere Daten wiesen nach, dass Mikrogliazellen, die durch niedrigdosierte PAMPs (LPS, β-Glucan) geprimt wurden, nach anschließender Exposition mit demselben oder einem anderen Erreger, erhöhte Produktion von pro-inflammatorischen Zytokinen zeigten (trainierten Sensibilisierung). Demgegenüber bildeten Mikrogliazellen nach Erstkontakt mit hochdosierten PAMPs einen robusten Toleranzphänotyp aus, wodurch nach Zweitkontakt mit dem gleichen oder einem anderen PAMP ein deutlich reduziertes proinflammatorisches Reaktionsmuster

resultierte und eine verstärkte entzündungshemmende Wirkung nachweisbar wurde. Die Induktion von trainierter Sensibilisierung und Toleranz wurde durch unterschiedliche metabolische Veränderungen (Glykolyse vs. oxidative Phosphorylierung) vermittelt. Darüber hinaus konnte nachgewiesen werden, dass bei Mikrogliazellen durch Lipidkinase-abhängige und -unabhängige Aktivitäten von PI3Kγ maßgeblich die Regulation des Zytokinprofils, metabolische Modifikationen und andere immun-relevante Zellfunktionen (z. B.Phagozytose-Aktivität) bewirkt werden.

I. Introduction

1.1. The pathogen dose as crucial determinant for memorizing enduring adaptive responses

"The dose makes the poison" (lat. *"Sola dosis facit venenum"*) is one of the fundamental dictums of Paracelsus, showing the importance of dose as crucial for the definition of its outcome in different organisms. Currently it was reported that innate immune cells are capable to mount memory-like responses, functionally in principal comparable with the immunological memory of the acquired arm after subsequent challenge (Kleinnijenhuis et al. 2012). Both of these adaptive responses, sensitization ("training") as a resistance mechanism aims to the reduction of the number of invading pathogens, and tolerance ("desensitization") which aims toward maintenance and repair mechanisms, were reported to be dependent on the nature of the stressors such as pathogen-associated molecular patterns (PAMPs) (Quintin et al. 2012). Epigenetic modifications imposed by the first hit with resulting changes in gene transcription and resulting metabolic changes rearrangements were disclosed as the basis for the development of a these adaptive memory-like effects in innate immune cells (Saeed et al. 2014).

Recent evidences have reported that priming of innate immune cells is shaped not only by the pathogen molecule, but also by the pathogen dose. Corresponding studies showed that monocytes sensitize priming with low-dose lipopolysaccharide (LPS), after repeated challenge inducing increased release of pro-inflammatory mediators such as cytokines (TNF-α, IL-6) and reactive oxygen species (ROS) (Chen et al. 2015; Yuan et al. 2016). Furthermore, priming with high-dose LPS induced prolonged reduction of inflammatory cytokines. Together these experiments identify the concentration of PAMPs as the critical mediator of trained sensitization and tolerance responses in monocytes, suggesting a general long-term (enduring) reaction pattern of innate immune cells that predominantly depends on the dose of conserved structures of different pathogens sensed by the cell (Bauer et al. 2018).

The above mentioned findings have been explained by different metabolic changes characterizing innate immune cells. Low amounts of different PAMPs provoke energy-demanding resistance responses aiming at pathogen elimination, serving as an adequate state to

maintain production of cytokines, antimicrobial peptides, and other defense mediators (Arts et al. 2016b). In contrast, high doses of pathogens increases impairing mechanisms by affecting the capacity of the cells to combat cytotoxic effects of the stressful stimulus triggering the shift from resistance responses toward maintenance and repair activities enabling tolerance responses against pathogens (Soares et al. 2014; Jha et al. 2015). Adenosine monophosphate-activated protein kinase (AMPK) and mechanistic target of rapamycin (mTOR) were reported as nodal points of intracellular signaling of both long-term adaptive responses, where mTOR promotes sensitization (training) with related metabolic changes, whereas AMPK is involved in maintenance effects associated with reduced LPS-induced of TNF-α release in macrophages (Cheng et al. 2014; Kim et al. 2014). Furthermore recent attempts were made in order to define the molecular mechanism behind innate immune adaptive responses induced by increasing loads of pathogen, where low doses of PAMPs or damage-associated molecular patterns (DAMPs) trigger the release of ROS and the direct or indirect activation of phosphoinositide 3-kinase (PI3K)/mTOR signaling pathway (Bauer et al. 2018).

Similar biphasic hormetic responses were reported also by other stressors such as heat, cold, toxins, radiation or either dietary restriction where low amounts induce anabolic processes mediated by mTOR signaling, whereas high doses activate AMPK and lead to maintenance operations and related catabolic reactions (Reiling and Sabatini 2006; Jeon 2016).

Taken together, these data underline the key importance of the sensing dose of stressors (here pathogens) for the development of innate immune-mediated adaptive responses. Thus we hypothesize a dose-dependent promotion of sensitization and tolerance by innate immune cells, such as microglial cells, as a fundamental principle for the pathogen induced adaptive response.

1.2. Trained sensitization and tolerance as new paradigms of the innate immunity

Years along the immune system in vertebrates has been categorized into two arms of immunity, the innate and the acquired immunity. The innate immune cells have been characterized to be the first line fighting invading pathogens, fast responding, non-specific and lacking on immunological memory. Innate host defenses are mediated by cellular and humoral responses involving cells such as monocytes, macrophages, neutrophils, natural killer (NK) cells, and the

brain resident cells- microglia as well as the complement system (Rivera et al. 2016). In the other hand the acquired immunity consists of specialized cells, T and B cells, which respond slowly, are specific and posses the ability to build the immunological memory against specific pathogens because of somatic hyper-mutation. The latter consists of accelerated somatic mutations and V(D)J recombination (an irreversible genetic recombination of antigen receptor gene segments) (Yancopoulos and Alt 1985).

In the past, perceived absence of immune memory of the innate immunity has been seen as a disadvantage considering them as evolutionary primitive. However, observations that children vaccinated with the Bacillus Calmette-Guérin (BCG) vaccine resulted in long-term decreased morbidity due to infections other then tuberculosis, was recognized to be a non-specific protection mechanism against pathogens mainly induced by innate immunity (Garly et al. 2003; Netea et al. 2011). Later on, studies done on macrophages and NK cells showed that priming of these cells with β-1,3-(D)-glucan (β-glucan) from C. albicans or with any viral ligands such as glycoproteins and double-stranded RNAs (dsRNAs) originating from respiratory syncytial virus (RSV), upon reinfection undergo a secondary expansion characterized with an increased pro-inflammatory cytokine profile and the induction of protective responses (Guillot et al. 2005; Sun et al. 2009; Ifrim et al. 2014).

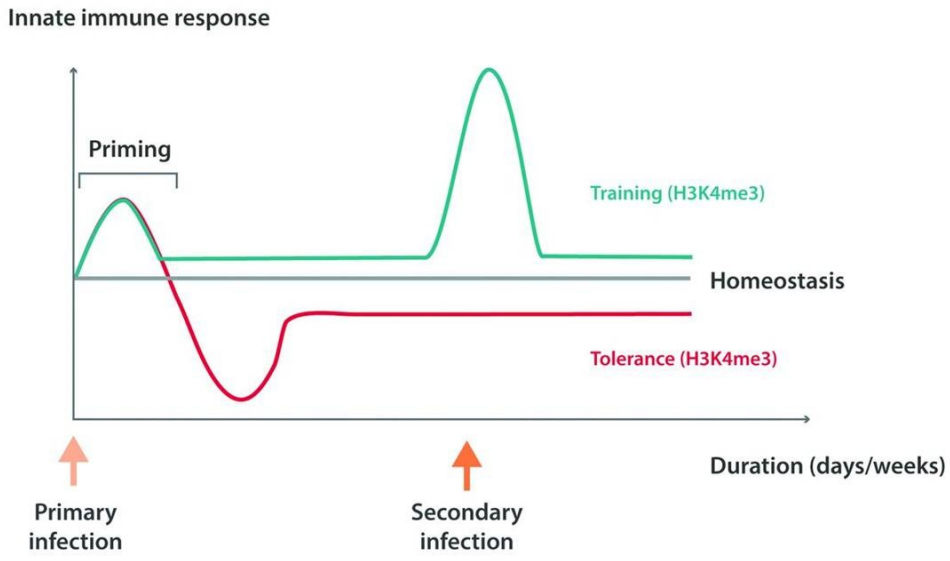

Figure 1: Trained sensitization and tolerance as memory-like activities in the innate immunity.
The illustration shows both of the adaptive responses of the innate immunity. Priming of the innate immune cells followed by a second challenge later on leads to elevated levels of pro-inflammatory profile

promoted by epigenetic modifications – enhanced trimethylation of histone H3K4 (H3K4me3) inducing trained sensitization. In contrast, some PAMPs like LPS, down-regulate the expression of the pro-inflammatory after subsequent challenge, inducing a tolerant response, characterized by the loss of H3K4me3- remaining silent upon restimulation. Adopted from (Stevens et al. 2016).

Taken together, it become clear that the innate arm of the immunity is also able to display memory-like properties named trained immunity (sensitization), different from the acquired immunity, characterized with enhanced levels of cytokine production (Netea et al. 2011).

In contrast, the phenomenon of endotoxin tolerance is defined as a shift toward an immuno-suppressive (anti-inflammatory) response, characterized by reduced inflammatory response of the innate immune cells after subsequent challenge with LPS (Cavaillon and Adib-Conquy 2006). Observations showed that animals are able to survive a lethal dose of bacterial endotoxin if they had been previously treated with a sublethal injection (West and Heagy 2002). Therefore, considering that uncontrolled systemic inflammatory response, induced by PAMPs such as LPS and mediated by a storm of pro-inflammatory cytokines resulting in a life threatening sickness syndrome including cardiovascular dysregulation (endotoxin shock), tissue damage, and others, thus (endotoxin) tolerance protects the affected organism by controlling (damping) the inflammatory state to prevent any hyper-inflammatory responses and serves as an important mechanism for host protection (Biswas and Lopez-Collazo 2009). The ability of the innate immune cells to induce trained sensitization and tolerance shows that the immune system in mammals represents an integrated continuum between innate and acquired immunity.

1.2.1. Trained sensitization in the innate immune cells

The dichotomy of innate and acquired immunity as components of the immune system represent not any sharp division as it was thought previously. The ability of innate immune cells to mount a memory-like behavior, functionally similar to the immunological memory of the acquired arm after subsequent infection has been termed as trained immunity (innate memory) (Netea and van der Meer 2017). These adaptive features can offer protection against reinfections by the same or different pathogens mediated independently of the classic acquired immunity (Sun et al. 2009; Paust et al. 2010).

Initial evidences of trained immunity in mammals have been reported with BCG vaccination and by priming the monocytes with β-glucan, as reported above (Ifrim et al. 2014). This contribution also pointed out a *pathogen-dependent* induction of adaptive mechanisms, where pathogen conserved components, PAMPs like β-glucan, BCG vaccine or modified lipoproteins such as oxidized low-density lipoprotein (oxLDL) or crystal hemozoin (Hz) of the malaria parasite (Plasmodium falciparum) - induce (in principle) trained sensitization characterized by enhanced levels of the pro-inflammatory response (e.g. TNF-α, IL-6) (Fig. 1) (Ifrim et al. 2014; Schrum et al. 2018).

Several findings have shown that trained immunity is mainly induced by epigenetic changes followed by metabolic modifications. A study showed that Dectin-1 activation by β-glucan during trained sensitization results with the activation of the protein kinase B (Akt)/mTOR pathway resulting with increased expression of HIF-1α, reflected with a metabolic shift toward aerobic glycolysis and characterized with enhanced levels of lactate production (Cheng et al. 2014; Arts et al. 2016b; Novakovic et al. 2016). They further reported that metformin, a mTOR inhibitor, was able to abolish the training effect induced by β-glucan through the Akt/mTOR pathway, mainly by indirect activation of AMPK, in addition to its capacity to combat C. albicans infection. Recently was shown that mediation of trained sensitization in monocytes is mainly induced by the metabolic changes, shifting toward glycolysis and glutamine metabolism, regulated by Akt/mTOR pathway and dependent on epigenetic reprogramming such as histone modifications (Arts et al. 2016a). Epigenetic reprogramming in the case of trained sensitization are characterized with methylation or acetylation of histones, leading to sustained changes in the transcription programs that do not involve permanent genetic changes such as mutations (Netea et al. 2016).

As mentioned above, trained sensitization has been mainly characterized by increased production of pro-inflammatory cytokines (such as TNF-α, IL-6, IL-1β) accompanied with the upregulation of specific histone marks that are involved in the transcription of this cytokine profile, such as trimethylation of lysine 4 residue on histone 3 (H3K4me3), methylation of lysine 4 residue on histone 3 (H3K4me1), and acetylation of lysine 27 residue on histone 3 (H3K27Ac) (Quintin et al. 2012; Crișan et al. 2016). β-glucan as main inducer of trained immunity has been further reported to reverse the immune tolerance state induced by the activation of Toll-like receptor 4

(TLR4) with LPS, restoring the ability of innate immune cells to mediate trained sensitization (Novakovic et al. 2016).

1.2.2. Endotoxin tolerance

Endotoxin tolerance was first described by Paul Besson early in the 20th century, where he was able to observe that repeated injections of typhoid vaccine caused an emerge reduction of the vaccine-induced fewer (Beeson 1947). Since then, multiple observations have shown that repeated injections of gram-negative bacteria result with decreased inflammatory response of innate immune cells provoking a process called endotoxin tolerance with a rather complex change in the sensitivity of these cells to pathogens (Gantner and Singh 2007; Foster et al. 2007). It was confirmed that LPS, as a major component of the gram-negative bacteria wall is the main and the strongest known inducer of the endotoxin tolerance. LPS as a PAMP, is recognized by TLR4, leading to the activation of downstream molecules that trigger the activation of nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NFκB), resulting with the production of inflammatory cytokines (e.g. TNF-α, IL-6, IL-1β) (Ghosh and Karin 2002). LPS-induced tolerance is an active process mediated by epigenetic changes such as the loss of H3K4me3 and promotion of trimethylation of lysine 9 residue on histone 3 (H3K9me3) resulting in a reduced pro-inflammatory response upon re-challenge (Fig. 1) (Wen et al. 2008; Ifrim et al. 2014; Doxaki et al. 2015).

Interestingly, when tolerance is induced by LPS, the respective innate immune cells enhance the phagocytic capacity as one of the main functions to fight against pathogens (De Lima et al. 2014). Furthermore, repeated challenge of human mononuclear cells with LPS induce endotoxin tolerance, leading to the known immunosuppressive responses characterized by decreased levels of pro-inflammatory mediators (TNF-α, IL-6) and increased anti-inflammatory cytokines such as IL-10, resulting in a switch toward the M2 state of polarization (Pena et al. 2011). Endotoxin tolerance is further characterized by metabolic alterations toward oxidative phosphorylation, mainly mediated by increased adenosine triphosphate (ATP) production that promote repairing mechanisms (Fensterheim et al. 2017).

The molecular mechanism behind endotoxin tolerance is yet not fully elucidated and incompletely understood. Obviously, during endotoxin tolerance a reduction of the pro-inflammatory cytokine profile occurs, which promotes the IL-10 or TGF-β production, respectively as anti-inflammatory mediators. Furthermore, LPS-induced tolerance is characterized by downregulation of TLR4/MyD88 pathway and partially promotion of TLR4/TRIF pathway leading to downregulation of pro-inflammatory mediators, such as TNF-α, IL-6 and iNOS, and resulting in increased levels of IL-10 and TGF-β (Nomura et al. 2000). Therefore endotoxin tolerance serves as an important control mechanism to attenuate inflammation-associated damages, leading to cellular re-arrangements affecting different functional processes in the organism (Chu et al. 2016).

1.2.3. Functional importance of trained sensitization and tolerance

The ability of the innate immune cells to respond with trained sensitization and tolerance is considered to be a new powerful tool for the development of new potential strategies against different diseases, especially those who imply inflammatory processes such as microbial infections, cardiovascular disease (such as atherosclerosis), neurodegenerative disorders and even malignant tumors (Dubois 2015; Norata 2018). Furthermore, understanding the molecular mechanism behind trained sensitization and tolerance may help for the development of new specific inhibitors, modifiers of the epigenetic reprofiling or metabolic pharmacological supplements that could be used for potent treatments of different disorders (van der Meer et al. 2015). Indeed, hyperactivation of the innate immune cells can lead to maladaptive responses promoting a pro-inflammatory setting resulting with progression of the chronic inflammatory diseases such as atherosclerosis (Becker et al. 2010; Bekkering et al. 2013).

Increased attention has been attracted toward current findings that alterations of neuro-inflammatory responsiveness associated with microglial trained sensitization and tolerance are crucially involved in the progression and regression of neurodegenerative disorders. Recent investigations showed that induction of trained sensitization in microglial cells trigger the exacerbation of the neuropathological diseases (i.e. Alzheimer disease) (Wendeln et al. 2018). Similarly, long-term hyperactivation and extensive release of pro-inflammatory mediators seems

to affect emotional, behavioral and cognitive properties by inducting neuro-inflammatory responses (Talge et al. 2007; Salam et al. 2018).

Trained sensitization represents therefore a double-edged sword in several functions of the innate immunity, because it can contribute to a beneficial host pathogen response, but can also aggravate the severity of chronic diseases.

1.3. Microglial cells – role and function in health and diseases

Microglial cells are long-lived, immunocompetent and resident cells of the central nervous system (CNS) that are involved in the maintenance, pathogenesis and repair mechanisms in the brain (Streit 2002; Harry and Kraft 2012). Microglial cells originate from early development of myeloid progenitors in the embryonic yolk sac and account for around 10% of the cells in the central nervous system (Lawson et al. 1990). They represent the brain macrophages, responsible for surveying the microenvironment in order to warrant homeostatic conditions for normal neural functions (Aguzzi et al. 2013). In any case of tissue disturbance, immediate microglial response contributes effectively in elimination of invading pathogens, damaged cells or protein aggregates, as well as dysfunctional synapses and antigens in the CNS (Amor et al. 2010). Therefore, any pathological event or injury in the CNS lead to the activation of the microglial cells that start to proliferate, migrate to the place of injury, become active phagocytes and remove debris or dead cells as well as dysfunctional synapses, a process termed “synaptic stripping”, affecting or reconstructing structural circuits in the neural network (Saijo and Glass 2011; Kettenmann et al. 2013).

Beside their crucial role in homeostatic maintenance, microglial cells are implicated in relevant functional alterations of brain development and aging (Luo et al. 2010). Furthermore, they have a pivotal role in the generation and progression of the neurodegenerative diseases (Zhang et al. 2017). Microglial cells produce a wide range of factors important for the maintenance of brain homeostasis and defense including chemokines, cytokines and neutrophic factors that trigger either an inflammatory cascades or acquire neuroprotective mechanisms that contribute to various aspects of immune responses and tissue repair in CNS (Pocock and Kettenmann 2007; Colonna and Butovsky 2017). The phenotype of these cells has been characterized based on the

morphological features. Seminal *in vivo* studies pointed out that there is no period of inactivity for microglial cells since they constantly scan the brain microenvironment in order to detect minor perturbations (Nimmerjahn et al. 2005; Kettenmann et al. 2011; Davis et al. 2017). Moreover under surveying state microglial cells have a highly ramified (branched) morphology and are able to survey the intact brain by extending and retracting their ramified motile processes while constantly contacting synapses (Nimmerjahn et al. 2005). Different stressors act on surface receptors of the microglial cells and rapidly activate them leading to morphological (amoeboid microglia) and functional changes like secretion of different mediators (i.e. chemokines, cytokines) that play a pivotal role in initiation and development of neuro-immunological processes (Hanisch 2002).

Microglia as the main innate immune cells has been also considered as double-edged sword, considering the fact that in one hand it can help eliminating pathogens and debris that could be harmful, but in the other hand "hyper"-activated microglia can cause severe neurodegenerative diseases (Kim and Joh 2006; Santiago et al. 2017). Therefore, an inhibition of "hyper"-activated microglia has been considered as a possible key strategy for the prevention and treatment of different neurodegenerative diseases like Alzheimer's diseases, Morbus Parkinson or multiple sclerosis, even if there is no approved treatment option so far (Dheen et al. 2007). Moreover, chronic activated microglia induces a pronounced phagocytic effect and remains as one of the main sources for multiple neurotoxic factors, including here tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), nitric oxide (NO) derived from inducible NO synthase (iNOS), interleukin-1-beta (IL-1β), and reactive oxygen species (ROS), that drive the progression of the neural damage (Lull and Block 2010). One of the observations shows that extensive production of ROS and increasing microglial phagocytosis (part of microgliosis) have been crucially involved in the mechanism of neurotoxic microglial activation, especially in the Parkinson disease (Roy et al. 2008).

Most of the immune receptors in the surveying microglia are quite low expressed, but upon activation of the microglia, these receptors are significantly upregulated and stimulate the production of inflammatory mediators (Walter and Neumann 2009). One of the most important receptor entities that leads to the activation of microglial cells are the family of pattern-recognition receptors (PRRs) that recognize different conserved pathogen structures (motifs)

from microorganisms termed PAMPs or endogenous molecules that originate from stressed cells upon tissue damage known as DAMPs (Häcker et al. 2006).

Different PAMPs activate PRRs and thus affect the PI3Ks/mTOR pathway leading to attenuated levels of pro- and anti-inflammatory cytokines in the microglial cells (Weichhart et al. 2015). Furthermore, previous studies showed that PI3Kγ, the only member of class I_B of PI3Ks, is constitutively expressed in microglial cells mediating different microglial functions such as migration, phagocytosis, ROS formation and MMP release (Jin et al. 2010; Schmidt et al. 2013; Frister et al. 2014; Schneble et al. 2017).

1.3.1. Pattern-recognition receptors (PRRs) as key activators of microglia

Microglial cells as a part of the innate immune system represent the first line of host defense against pathogens. They have been considered to be unspecific and transmit the information as antigen-presenting cells to the acquired immune system. Up to date, it was shown that the innate immune cells are not completely nonspecific, as was originally thought, but rather are able to discriminate between self and a variety of pathogens (Akira et al. 2006). They recognize different pathogens by sensing them via distinct PRRs leading to the activation and the production of pro-inflammatory cytokines.

PRRs represent a germline-encoded receptor family that posses the ability to recognize microbial components, known as PAMPs, that are essential for the survival of the microorganism and are therefore difficult for the microorganism to alter (Nhu et al. 2012). In addition, PRRs also recognize different host biomolecules called DAMPs (Wegiel et al. 2015; Hooper et al. 2018). Up to date there are four major families of PRRs present in the innate immune cells: Toll-like receptors (TLRs) with about 13 murine, respectively 10 human identified members; RIG-like receptors (RLRs); NOD-like receptors (NLRs); and C-type lectin receptors (CLRs) with several members including here Dectin-1 which is able to recognize fungal components like β-glucan (Medzhitov 2007).

TLRs recognize a variety of different PAMPs with diverse origins (viruses, bacteria, fungus, and parasites). They are characterized by an extracellular N-terminal leucine-rich repeats (LRRs) domain and a transmembrane region followed by a cytoplasmic Toll/IL-1R homology (TIR)

domain (Akira and Takeda 2004; Takeuchi and Akira 2010). Members of TLRs family can further be divided based on their recognition preferences for different PAMPs, like them that recognize lipids from bacteria (TLR1, TLR2, TLR6) or nucleic structures from viruses (TLR7, TLR8, TLR9) (Janeway and Medzhitov 2002). Activation of Toll-like receptors causes activation of cascade proteins that induce the translocation of NFκB into the nucleus resulting with expression of pro-inflammatory genes and production of different cytokines (of major interest TNF-α and IL-6).

One of the most important receptors of TLRs is TLR4 which recognizes a range of ligands like LPS, fibronectin, and heat-shock proteins of different structures (Akira et al. 2006). LPS acts through TLR4 with the help of the anchor protein, myeloid differentiation factor 2 (MD-2), and activates two divergent pathways: Myeloid Differentiation Factor 88 (MyD88)-dependent and MyD88-independent pathway.

MyD88-dependent pathway is mainly characterized to transmit the activation response through the adaptor protein MyD88 triggering the early activation of the transcription factor NFκB and thus leading to the production of inflammatory mediators (Kawai et al. 2001). Different, MyD88-independent pathway leads to the activation of late phase NFκB and interferon regulatory factor 3 (IRF-3) as transcription factors triggering the production of interferon-beta (IFN-β) and pro-inflammatory cytokines through transmission of the signal via TRIF (Toll/IL-1 receptor (TIR) domain-containing adaptor) as an adaptor protein (Yamamoto et al. 2003). Moreover NFκB is not only the prototypical pro-inflammatory signaling pathway but also a key regulator of the anti-inflammatory response that regulates the expression of pro- and anti-inflammatory mediators, chemokines, and adhesion molecules (Lawrence 2009).

Dectin-1 receptor as a member of CLRs, is mainly activated from β-glucan and recruits downstream regulation of caspase recruitment domain-containing protein 9/ B-cell lymphoma-leukemia 10/ Mucosa-associated lymphoid tissue lymphoma translocation protein 1 (CARD9/BCL10/MALT1) complex triggering the activation of NFκB, expressing increased production of TNF-α and IL-6 (Plato et al. 2015). Therefore, pathogen-induced upregulation of PRRs and related signaling molecules by microglia in the CNS indicates a prominent role in neurodegeneration and repairing mechanisms (Kigerl et al. 2014).

1.3.2. Different functional activation states of microglial cells

Microglial cells under healthy conditions have a wide range of important functions such as synaptic pruning during development shaping neural circuits in the developing brain, promoting the secretion of neutrophic factors such as such as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) thus contributing to the formation of learning-dependent synapses or by secretion of cytokines supporting the process of neurogenesis (Shemer et al. 2015; Butovsky and Weiner 2018). Pathological events trigger the activation of microglia, a predominant process that induces neuroinflammation in the central nervous system (Glass et al. 2010). Under the influence of different stimuli *in vitro*, microglia tends to show a polarized phenotype into a pro-inflammatory (M1) classical activated form or anti-inflammatory (M2) (regenerative) alternative activation. Despite all the characteristics between M1 and M2 phenotypes, increasing evidence has shown that this kind of arrangement has been helpful for conceptualizing microglia responses *in vitro*, but doesn't represent the *in vivo* activation where microglial cells rarely display any bias toward any of these states (Ransohoff 2016; Colonna and Butovsky 2017). Results from single-cell RNA-sequencing analysis of the infiltrating brain macrophages during traumatic brain injury (TBI) failed to support the M1/M2 polarization scheme, suggesting a lack of exclusivity in macrophage polarization states *in vivo* (Kim et al. 2016). In addition an another study showed also that during brain injury, macrophages/microglia in a concurrent manner express a mixed phenotype, different from the common know polarization phenotypes (Morganti et al. 2016).

Under *in vitro* conditions, M1 activation represents a pro-inflammatory and neurotoxic state induced by LPS or other ligands that trigger the downstream stimulation of TLR pathway, inducing the production of a range of pro-inflammatory cytokines such as TNF-α, IL-6, and IL-1β (Kobayashi et al. 2013). The outcome of M1 microglia includes also high production levels of reactive oxygen species (ROS), as well as the inducible nitric oxidase (iNOS) which mainly competes with arginase-1 (Arg-1) as M2 mediator, for the metabolism of L-arginine (Orihuela et al. 2016). Furthermore, iNOS converts L-arginine into nitric oxide (NO) and citruline having a significant impact in the inflammatory state of microglia. In addition, classic activation of microglia is characterized with a metabolic switch toward a glycolytic state, driving a sustained expression of hypoxia-inducible factor 1-alpha (HIF-1α) (Habib et al. 2014; Van den Bossche et

al. 2016). M1 state plays a crucial role responding to injury and it represents the first line against invading pathogens in the CNS.

In contrast, the alternative (M2) activation of microglia promotes the anti-inflammatory state and repairing mechanisms induced mainly by IL-4 stimulation. Upon activation, M2 phenotype leads to the release of the anti-inflammatory cytokines like IL-10 and TGF-β, neurotrophic factors like BDNF, as well increased expression of Arg-1 that constitutively competes with iNOS for L-arginine metabolism, making it a key factor for the differentiation between M1/M2 phenotype activation (Pesce et al. 2009; Avdic et al. 2013; Tang and Le 2016; Taylor et al. 2017). Arg-1 converts L-arginine into urea and L-ornithine which promotes cell proliferation and tissue repair (Landau et al. 2010). Distinct from M1, the M2 microglia are characterized with a pronounced oxidative phosphorylation as metabolic change. Furthermore, the M2 phenotype promotes parasite clearance, immunosuppression, wound healing and tumor growth (Murray and Wynn 2011). The M1/M2 polarization concept has been oversimplified in order to explain different characteristics of microglial cells, but failed to yield a clear-cut meaningful explanation of the transcriptional profile and don't represents any specific relation to any cellular functions in microglia (Grabert et al. 2016).

1.4. PI3Ks family - key signaling proteins of different immuno-responses

Phosphoinositide 3-kinases (PI3Ks) represent a family of lipid kinases that transduce signals from transmembrane receptors such as receptor tyrosine kinases (RTKs) and G protein-coupled receptors (GPCRs) to the cytoplasm and results in production of phosphorylated lipids (Akinleye et al. 2013). They phosphorylate phosphatidylinositol (PtdIns) and its phosphorylated derivatives at the 3′-OH group position of the inositol ring, generating second messengers that control and modulate intracellular activities of protein effectors affecting many cellular functions (Bader et al. 2005; Martini et al. 2014). PI3Ks control a wide range of cell functions including survival, migration, proliferation, metabolism, cell growth and immune responses. Taking under consideration that this family of lipid kinases can monitor such a big spectrum of biological processes, it is of key importance to study them specifically. Based on the structural similarities and substrate specificities, PI3Ks are divided in three classes: class I, further divided in class I_A

(enzymes: p110α, p110β, and p110δ) and class I_B with the only member p110γ; class II with 3 identified catalytic members (C2α, C2β, and C2γ), and class III with the only one known member vacuolar protein sorting 34 (Vps34) (Liu et al. 2009).

Class I_A is the most studied class of PI3Ks, consisting of a regulatory subunit (p85α, p85β or p55γ) and a catalytic subunit (p110α, p110β or p110δ) where each member can associate with all of the regulatory subunits (Okkenhaug and Vanhaesebroeck 2003). Upon stimulation the regulatory subunit binds to the intracellular phosphorylated tyrosine motifs of the activated receptor and triggers the activation of the catalytic subunit mediating the phosphorylation of phosphatidylinositol-4,5-bisphosphate (PIP_2) into phosphatidylinositol-3,4,5-trisphosphate (PIP_3) which serves as second messenger recruiting the activity of class I PI3Ks (Bader et al. 2005; Martini et al. 2014). PIP_3 as a secondary messenger acts as a binding site for numerous intracellular enzymes that contain PH (pleckstrin homology) domains, especially for serine-threonine kinase Akt known also as protein kinase B (PKB), as well 3-phosphoinositide-dependent kinase 1 (PDK1) (Okkenhaug and Vanhaesebroeck 2003; Bader et al. 2005; Yu and Cui 2016). Akt is one of the main downstream effectors of PI3Ks that activates effectively the mechanistic target of rapamycin complex 1 (mTORC1), glycogen synthase kinase 3 (GSK3), tuberous sclerosis complex (TSC), and the forkhead family of transcription factors (FOXOs) resulting in increased protein synthesis, metabolism and proliferation (Zhang et al. 2006). PIP_3 serves also as a substrate for phosphatase and tensin homolog (PTEN), which dephosphorylates PIP_3 into PIP_2 and thus results as a negative regulator of PI3K/Akt signaling and functions (Malek et al. 2017). Due to pivotal functions of class I_B of PI3Ks in the immunologic responses in the innate immune responses, especially in microglial cells it will be described in special parts below, elaborating crucial cellular functions of the only member, phosphoinositide 3-kinase γ (PI3Kγ).

Class II are monomers that lack on the regulatory subunit binding domains, but possess three catalytic isoforms: C2α, C2β, and C2γ (Falasca and Maffucci 2012). They are activated through both RTKs and GPCRs, and considered for years long as very enigmatic involved in cell growth, vesicular trafficking, and migration (Gaidarov et al. 2001; Falasca and Maffucci 2012; Mavrommati et al. 2016). Vps34 is the only identified member of class III of PI3Ks, known to be involved in vesicular trafficking and nutrient sensing in the mTOR pathway in mammalian cells

(Devereaux et al. 2013). Considering all the above mentioned functions triggered by PI3Ks, especially involved in the cancer, there are increasing attempts for discovering new anti-cancer therapies by different inhibitors that suppress specifically class I PI3Ks (Zhao et al. 2017).

1.4.1. Class I_B of PI3Ks

Class I_B of PI3Ks are heterodimers of a p110γ catalytic subunit coupled with the regulatory isoforms p101 or p87 (also known as p84 or $p87^{PIKAP}$) (Thorpe et al. 2015). PI3Kγ as the only member of this class is activated by GPCRs, by the interaction of regulatory subunit with Gβγ, triggering the rapid phosphorylation of PIP_2 into PIP_3 as second messenger and thus activating Akt/mTOR downstream processes. Several chemokines (e.g. IL-8), bacterial products (e.g. N-formyl-Met-Leu-Phe (fMLP), LPS) and complement system factors (e.g. C5a) have been shown to be able to activate this isoform of PI3Ks and thus regulating cellular functions of the immune cells from both innate and acquired immunity (Hawkins and Stephens 2007). PI3Kγ is involved in stress-induced inflammatory responses and highly expressed in neutrophils, macrophages, mast cells, as well as important due to many reported functions in microglia, T cells, endothelial cells (Hawkins et al. 2006; Jin et al. 2010), but also parenchymal cells like cardiomyocytes and neurons of the central as well as peripheral nervous system (Patrucco et al. 2004; König et al. 2010; Perino et al. 2014).

Chemoattractants interact with GPCRs and activate PI3Kγ resulting with increased production of ROS in order to kill the pathogen and leading to inflammatory response (Fruman et al. 2017). TLRs recognize different pathogen conserved structures, thus tightly affecting PI3Ks/mTOR pathway and attenuating the production of pro- (TNF-α, IL-6, IL-1β) and anti-inflammatory (IL-10) cytokines (Weichhart et al. 2015). Moreover PI3Kγ represents a crucial mediator of inflammatory responses, especially in cardiomyocytes and microglial cells (Ndongson-Dongmo et al. 2015; Schmidt et al. 2016).

PI3Kγ triggers the phosphorylation of PIP_2 into PIP_3 as a second messenger activating different downstream effectors, most importantly Akt and PDK1. Furthermore, PI3Kγ has also been shown to be directly activated by Ras, controlling MAPK signaling pathway, such as Jun-N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK) cascades (Rückle et al.

2006). Moreover, mice lacking PI3Kγ had significantly decreased release of pro-inflammatory cytokines and increased protection from septic induced disorders (Vadas et al. 2013). A recent work showed that PI3Kγ is a key switch molecule that controls immunosuppression affecting NFκB activation, and synergizes with inhibitor therapy to promote long-term tumor regression and increased survival (Kaneda et al. 2016).

PI3Kγ has been reported to control immuno-metabolic responses in the innate immune cells by regulating diet-induced obesity, adipose tissue inflammation, and insulin resistance (Breasson et al. 2017, 2018).

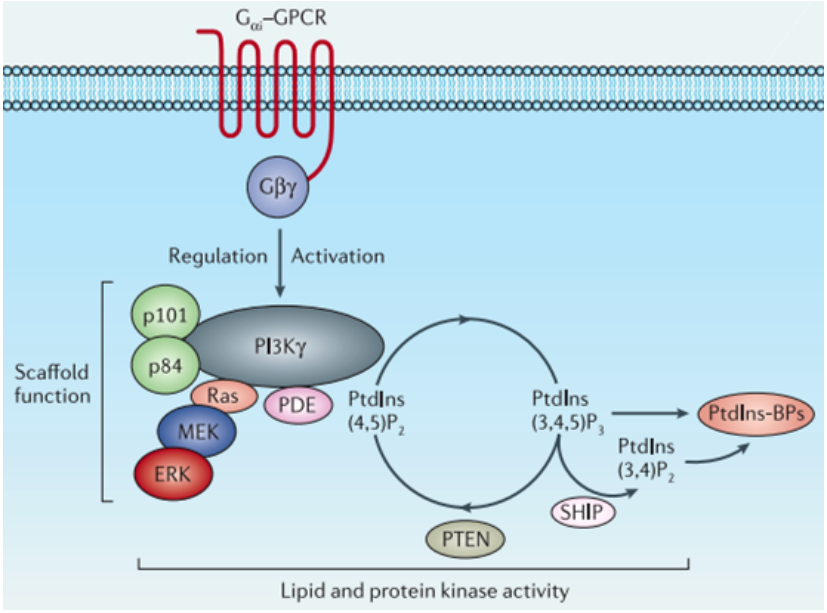

Figure 2: Dual functions of PI3Kγ, lipid kinase-dependent and -independent.
Gβγ subunits of G-protein coupled receptors (GPCRs) activates PI3Kγ triggering the phosphorylation of PIP_2 (phosphatidylinositol-4,5-bisphosphate) into PIP_3 (phosphatidylinositol-3,4,5-trisphosphate) and activating downstream processes which represents the catalytic activity (lipid kinase-dependent). In the other hand PI3Kγ binds to proteins like the regulatory subunit (p101 and p84), PDE (phosphodiesterase), Ras and mitogen-activated protein kinase (MAPK) kinase (MEK) mediating a scaffold-function (lipid kinase-independent). PTEN (phosphatase and tensin homologue) dephosphorylates PIP_3 to PIP_2. Adopted from (Rückle et al. 2006).

Interestingly, PI3Kγ consists of a dual role (so called "double identity"), not just acting like classical kinases by generating PIP_3 and inducing downstream signaling processes, but also binding with other proteins, especially PDE, indicating a protein-scaffold function in addition to its enzymatic activity as shown (Fig. 2) (Rückle et al. 2006; Costa and Hirsch 2010). The attempting step to differ and show both of those dual functions of PI3Kγ have been made by

using the knock-out (PI3Kγ$^{-/-}$) and the mutated form knock-in (kinase-dead; PI3Kγ$^{KD/KD}$) where the kinase activity has been abolished allowing only the lipid kinase-independent function to act (Patrucco et al. 2004). They showed that PI3Kγ$^{KD/KD}$ mice did not alter the cardiac contractility, but displayed in addition reduced inflammatory responses. A study from our group has showed that the scaffold-function (lipid kinase-independent) induced myocardial depression by sequential activation of cAMP and iNOS pathways (Ndongson-Dongmo et al. 2015).

Altogether, these studies underlined the tight interplay between lipid kinase-dependent and -independent functions of PI3Kγ, providing a potentially wider set of associations and functional relevance.

1.4.2. PI3Kγ role and function in microglia

There is growing evidence that PI3Kγ plays a pivotal role in controlling a variety of immune functions in microglial cells. Frister et al. showed that PI3Kγ lipid kinase-independent function mediated LPS-induced extensive matrix metalloproteinase 9 (MMP-9) production in microglial cells contributing to the breakdown of the blood-brain barrier (BBB) and thus inducing a neuro-inflammatory response and subsequently being a key event for the development of sepsis-induced brain damage (Frister et al. 2014). The data suggested that the PI3Kγ lipid kinase-independent stimulation of PDE (mainly PDE3B) activity resulting in an inhibition of cAMP signaling is a key mediator of LPS-induced MMP release in microglial cells. Another study of our group indicated the important scaffold function of PI3Kγ mediating microglial phagocytic activity after focal brain ischemia controlled by a PI3Kγ-dependent cAMP signaling (Schmidt et al. 2013, 2016). The suppressive effect of PI3Kγ on cAMP levels through the activation of PDEs, especially PDE3B, appears to be critical for the restriction of ischemia- induced immune cell functions and in turn tissue damage (Schmidt et al. 2016). Furthermore, the lipid kinase-dependent activity of PI3Kγ that leads to the activation of Akt signaling events is an essential mediator of microglial migration and motility provoked by the complement component C5a and other chemoattractants (Schneble et al. 2017). Feed-back action of protein kinase A (PKA) induced by norepinephrine leads to the inhibition of PI3Kγ activity suppressing the cellular activity of migration (Schneble et al. 2017).

Therefore, lipid kinase-dependent and -independent activities of PI3Kγ have been shown to have either a coordinated or self actions in different downstream effectors affecting different cellular functions in microglial cells. Current findings regarding targeted metabolic control, mediated by activities of PI3Ks/mTOR pathway, suggest a possibly importance of PI3Kγ in mediating trained sensitization and tolerance in microglial cells.

II. Hypothesis and aims

2.1. Hypothesis

The ability of the innate immune cells to respond with trained sensitization and tolerance shows that the immune system in mammals represents an integrated continuum between innate and acquired immunity (Ifrim et al. 2014). Trained sensitization aims within the target to reduce the number of pathogens as protective mechanism, whereas tolerance in the other side affords protection against deleterious effects of infectious diseases as a maintaining mechanism (Bauer et al. 2018).

A previous report claimed that microglial cells are capable to induce tolerance response after repeated LPS stimulation (Schaafsma et al. 2015). Recently, a study observed dose-dependent correlation for the induction of both of adaptive immuno-responses after repeated challenge in microglial cells *in vivo*, displaying the key importance of pathogen dose, especially PAMPs such as LPS (Wendeln et al. 2018). Our research group observed dose-dependent effects after single stimulation affecting different cellular processes in microglial cells such as phagocytosis, migration, proliferation and cytokine release, mediated mainly by lipid kinase-dependent or -independent functions of PI3Kγ (Schmidt et al. 2013; Frister et al. 2014; Schneble et al. 2017). However, the role of different stressors such as LPS and β-glucan to induce adaptive responses (trained sensitization and tolerance) in microglia upon re-challenge has not been fully elucidated, especially the role of PI3Kγ controlling them.

Considering the above mentioned findings we hypothesized that different PAMPs such as LPS and β-glucan, are able to induce trained sensitization and tolerance responses in a dose-dependent manner in microglial cells. We further proposed that both, trained sensitization and tolerance, in microglia are mediated by the lipid kinase-dependent and/or -independent activities of PI3Kγ.

In addition we postulated that PI3Kγ regulates different metabolic parameters affecting major energy changes that are key developments during adaptive responses and influences cellular functions in the microglial cells (e.g. phagocytosis).

2.2. Aims

Taking into consideration the above mentioned reports and data we hypothesized a tight regulation of trained sensitization and tolerance in microglial cells by PI3Kγ. Our current aims for the project were:

- Deciphering the dose-dependent induction of both of adaptive responses on microglial cells, especially low-dose induction of resistance (trained sensitization) and high-dose induction of persistence (tolerance).
- Characterization of M1-like and M2-like microglia phenotypes as putative mechanisms helpful for conceptualizing immune responses *in vitro*, in order to explain trained sensitization and tolerance as new paradigms of the innate immunity in the CNS.
- To investigate the role of metabolic changes affecting different energy parameters in microglial cells important for the induction of trained sensitization and tolerance responses, mainly regulated by the activity of PI3Kγ.
- To characterize the molecular mechanism behind trained sensitization and tolerance responses in microglial cells mediated by lipid kinase and scaffold activities of PI3Kγ.
- To elucidate the role of PI3Kγ in different microglial functions, exclusively the phagocytic activity, during both of adaptive responses.

III. Materials and methods

3.1. Materials

Table 1: Machines and equipments

Machine/ Equipment	Manufacturer
Bio-Rad 1000/500 Constant Voltage Power Supply	Bio-Rad, USA
Centrifuge 5415C Eppendorf	Eppendorf AG, Germany
Centrifuge 5416 Eppendorf	Eppendorf AG, Germany
Centrifuge 5417R Eppendorf	Eppendorf AG, Germany
CKX41 Inverted Microscope	Olympus Microscopy, Japan
Cold Light Schott KL 750	Schott AG, Germany
CX41 Biological Microscope	Olympus Microscopy, Japan
HERAcell vios 160i (cell culture incubator)	Thermo Scientific, Germany
Lab pH Meter inoLab® pH 7110	Xylem Analytics Germany Sales GmbH & Co. KG, WTW, Germany
LAS4000 camera	Fuji Photo Film Co., Japan
Nanodrop ND-1000 machine	Peqlab Biotechnologies, Germany
Neubauer Counting Chamber	Carl Zeiss AG, Germany
Nikon Eclipse Ti fluorescence Microscope	Nikon Instruments, Japan
Pipette Pipetman Gilson (P10, P20, P100, P200, P1000)	Gilson S.A.S., France
Realplex Mastercycler ep gradient S	Eppendorf AG, Germany
Safe 2020 Class II Biological Safety Cabinet (cell culture safety bench)	Thermo Scientific, Germany
Sartorius Analytical Balance	Sartorius AG, Germany

Sartorius Basic Balance BA210	Sartorius AG, Germany
Seahorse XFe96 Analyzer	Agilent Technologies, Germany
Semi-Dry-Blotting Machine	Millipore Graphite, Millipore USA
Shaker type VF2	IKA-Labortechnik, Germany
Synergy® UV Water Purification System	Merck Millipore, USA
Systec Autoclave D65	Systec GmbH, Germany
TECAN Infinite 200 Plate reader	Tecan, Switzerland
Thermo cycler T-Gradient ThermoBlock	Biometra, Germany
Thermo mixer 5436 Eppendorf	Eppendorf AG, Germany
Trans-Blot® SD Semi-Dry Transfer Cell	Bio-Rad, USA
Transferpette® -8 electronic, Multichannel pipette	Brand GmbH & Co. KG, Germany
Tuttnauer Systec 5050 EL	Tuttnauer Europe B.V., Netherlands
Tweezers	Carl Roth GmbH & Co. KG, Germany
VersaMax Microplate Reader	Molecular Devices, USA
Zeiss Stemi 2000 Microscope	Carl Zeiss Microscopy, Germany
Zeiss Telaval 31 Microscope	Carl Zeiss Microscopy, Germany

Table 2: General laboratory chemicals, kits, consumables and reagents

Product	**Vendor**	**Catalogue Number**
12 Well Cell Culture Plates (sterile, with lid)	Greiner Bio-one	665180
2,5% Trypsin (10x)	Gibco	15090-046
2',7'-Dichlordihydrofluorescein-diacetat (H_2DCFDA)	Thermo Fisher Scientific	D399

24 Well Cell Culture Plates (sterile, with lid)	Greiner Bio-one	662160
2-Mercaptoethanol	Fluka Biochemika	63690
6 Well Cell Culture Plates (sterile, with lid)	Greiner Bio-one	657160
96 Well Cell Culture Plates (sterile, F-bottom, with lid)	Greiner Bio-one	655180
ABTS	Sigma - Aldrich	A1888
Acetic acid	Carl Roth GmbH & Co. KG	3738.5
Amersham Hybond P 0.45 PVDF blotting membrane	GE Healthcare Bio-Sciences	10600023
Ammonium persulphate (APS)	SERVA Electrophoresis GmbH	13375.05
Amphotericin B solution	Sigma - Aldrich	1397-89-3
Biosphere Filter tips (100 µl)	Sarstedt AG & Co.	70.760.212
Bovine serum albumin (BSA)	Sigma - Aldrich	A7906
Bromophenol blue	Carl Roth GmbH & Co. KG	A512.1
Calcium chloride ($CaCl_2$)	AppliChem GmbH	A4689,0250
cAMP GloAssayKit	Promega	V1501
Cell Scraper	Greiner Bio-one	541070
ClearLine® Filter tips (200 µl)	Dominique DUTSCHER S.A.S. - Siège	014220CL
Dimethyl sulfoxide (DMSO)	Sigma - Aldrich	D2650
Dinatriumhydrogenphosphat Dihydrat	Carl Roth GmbH & Co. KG	4984.2
Disposable Scalpels Präzisa	P. J. Dahlhausen & Co. GmbH	1100000722
DNase I	AppliChem Panreac	A3778,0050
Dulbecco's Modified Eagle's Medium (DMEM) – high glucose	Sigma - Aldrich	D6429
Ethanol	Carl Roth GmbH & Co. KG	K928

Ethylenediaminetetraacetic acid (EDTA)	Carl Roth GmbH & Co. KG	8043.2
F96 MaxiSorp Nunc-Immuno Plates	VWR International	62409-024
Fetal Bovine Serum (FBS)	Sigma - Aldrich	F7524
Filter tip FT 1000 (G) (1000 µl)	Greiner Bio-one	740288
Filtropur S 0.2	Sarstedt AG & Co.	83.1826.001
First Strand cDNA Synthesis Kit	Thermo Fisher Scientific	K1612
Fluoromount-G®	Southern Biotech	0100-01
Formaldehyde Solution 5 %	Otto Fischar GmbH & Co. KG	27261
Glycerol	Carl Roth GmbH & Co. KG	3783.2
Glycine PUFFERAN	Carl Roth GmbH & Co. KG	3908.3
HEPES PUFFERAN	Carl Roth GmbH & Co. KG	9105.4
Horseradish Peroxidase (HRP)	Sigma - Aldrich	77332
Hydrochloric acid fuming 37%	Carl Roth GmbH & Co. KG	4625.1
Injekt® 20 ml	B. Braun Melsungen AG	4606205V
Ionic Detergent Compatibility Reagent (IDCR)	Thermo Fisher Scientific	22663
Isopropanol	Carl Roth GmbH & Co. KG	6752.4
Lactate Oxidase (LO)	Sigma - Aldrich	L0638
Lactate Standard for IC – TraceCERT	Sigma - Aldrich	07096
Leupeptin-hemisulphate	AppliChem GmbH	A2183,0025
Lipopolysaccharide (LPS) from E. coli serotype 055:B5	Sigma - Aldrich	L2880
Magnesium chloride ($MgCl_2$)	Carl Roth GmbH & Co. KG	HN03.1
Maxima SYBR Green/ROX qPCR Master Mix (2X)	Thermo Fisher Scientific	K0221

Menzel-Gläser – Cover slips (24 x 32 mm)	VWR International	631-0711
Menzel-Gläser Superfrost – Microscope slides (76 x 26 mm)	Thermo Fisher Scientific	AG00008032E
Micro tube 1,5 mL	Sarstedt AG & Co.	72.690.001
Micro tube 1.5ml SafeSeal	Sarstedt AG & Co.	72.706.400
Micro tube 2,0 mL	Sarstedt AG & Co.	72.691
Micro tube 2.0ml SafeSeal	Sarstedt AG & Co.	72.695.400
Micro tubes 1.5 mL	Sarstedt AG & Co.	72.690.001
Mouse IL-10 ELISA kit	BioLegend	431412
Mouse IL-6 ELISA kit	BioLegend	431302
Mouse TNF-α ELISA kit	BioLegend	430902
N,N,N',N'-Tetramethylethylenediamine (TEMED)	Serva Electrophoresis	35930.02
Nonylphenylpolyethylenglycol (NP)-40	Merck KGaA	492016
Nunc-Immuno™ MicroWell™ 96-Well Plates, Thermo Scientific	VWR International	442404
PageRuler™ Prestained Protein Ladder	Thermo Fisher Scientific	26616
Paraformaldehyde	Sigma Aldrich	158127
Pasteur pipettes	Karl Hecht "Assistent" GmbH	40567002
Pefabloc SC (AEBSF hydrochloride)	AppliChem	A1412,0500
Penicillin – Streptomycin	Sigma - Aldrich	P0781
Pepstatin A	AppliChem	A2205,0025
Pierce 660nm Protein Assay Kit	Thermo Fisher Scientific	22662
Pipette tips (10 - 200 µL)	Greiner Bio-one	739296
Pipette tips (200 - 1000 µL)	Greiner Bio-one	740290

Poly-L-Lysin	Sigma - Aldrich	P2636
Potassium acetate	Carl Roth GmbH & Co. KG	4986.1
Potassium chloride (KCl)	Carl Roth GmbH & Co. KG	6781.1
Potassium dihydrogen phosphate (KH_2PO_4)	Carl Roth GmbH & Co. KG	3904.1
Potassium hydroxide (KOH)	Fluka Chemie AG	60370
Powdered milk	Carl Roth GmbH & Co. KG	T145.3
Propidium iodide	EMD Chemicals	537059
QIAzol Lysis Reagent	Qiagen	79306
RNase Away	Molecular BioProducts	7003
Rotiphorese Gel 30 (30% Acrylamide/bis-acrylamide 37.5:1)	Carl Roth GmbH & Co. KG	3029.1
SafeSeal SurPhob Filter tips (10 µl)	Biozym Scientific GmbH	VT0210
Sapphire microplate 96 well for PCR	Greiner Bio-one	652250
Seahorse XF96 V3 PS Cell Culture Microplates	Agilent Technologies	101085-004
Sodium chloride (NaCl)	Carl Roth GmbH & Co. KG	3957.2
Sodium deoxycholate	Sigma - Aldrich	D6750
Sodium dodecyl sulphate (SDS) Pellets	Carl Roth GmbH & Co. KG	CN30.3
Sodium dodecyl sulphate (SDS) Sol. 20%	AppliChem	A3942,1000
Sodium Hydroxide (NaOH)	Sigma - Aldrich	9356.1
Sodium orthovanadate	Sigma - Aldrich	S6508
SurPhob Pipette tips (10µL)	Biozym Scientific GmbH	VT0110X
TC Dish 100, Standard	Sarstedt AG & Co.	83.3902
TC Flask T75, Standard, Vent. Cap	Sarstedt AG & Co.	83.3911.002

Thiazolyl Blue Tetrazolium Bromide	Sigma - Aldrich	M5655
TMB Substrate Set	BioLegend	421101
Transfer pipette 3.5 mL	Sarstedt AG & Co.	86.1171
Trichlormethan/ Chloroform	Carl Roth GmbH & Co. KG	3313.1
Tris ultrapure	AppliChem	A1086,5000
Tubes, 15 mL	Greiner Bio-one	188271
Tubes, 50 mL	Greiner Bio-one	227261
Tween 20	Serva Electrophoresis	3747.0
Western Lightning Plus-ECL, Enhanced Chemiluminescent Substrate	PerkinElmer	NEL104001EA
Zymosan A (S. Cerevisiae) BioParticles, fluorescein conjugate	Thermo Fisher Scientific	Z2841
β-1,3-(D)-glucan from C. Albicans	Provided (and produced) by David Williams, East Tennessee State University, USA	

Table 3: Primary antibodies

Antibody	**Vendor**	**Cat. Number**	**Application**
iNOS	Abcam	ab3523	Western blotting
NFκB p65 (D14E12)	Cell Signaling Technology	8242	Western blotting
p100γ	Institute for Molecular Cell Biology, Jena - Germany		Western blotting
P-NFκB p65 (S536)	Cell Signaling Technology	3033	Western blotting
Rel B	Cell Signaling Technology	4954	Western blotting
TNF-α	Cell Signaling Technology	3707	Western blotting
β-actin	Sigma Aldrich	A2228 A5441	Western blotting

Table 4: Secondary antibodies

Antibody	Vendor	Type	Dilution	Application
HRP- anti mouse	KPL	goat polyclonal	1:10.000	Western blotting
HRP- anti rabbit	KPL	goat polyclonal	1:10.000	Western blotting

Table 5: Antibodies for Immunohistochemistry

Antibody	Vendor	Cat. Number	Dilution
Alexa Fluor® 568 donkey anti-goat IgG	Thermo Fisher Scientific	A-11057	1:250
Goat Iba-1	Abcam	ab5076	1:250

3.1.1. General buffers and solutions

Phosphate-buffered saline (PBS)

PBS (containing 8 g/L NaCl, 0,2 g/L KCl, 1,42 g/L Na_2HPO_4, 0,24 g/L KH_2PO_4) solved in double-distilled water ($_{dd}H_2O$) and the pH value has been adjusted to 7,5 and thereafter autoclaved.

TE buffer

All the primers (Table 6) were provided dry and ready for use upon resuspension with a weak buffer such as TE buffer (10 mM Tris, 1 mM EDTA, pH adjusted to 7,5) in a stock solution of 100 µM and stored at -20°C. For real-time experiments a concentration of 10µM was suitable.

3.2. Methods

3.2.1. Preparation and cell culture of primary microglial cells

Primary microglial cells have been isolated from the brains of the newborn mice (P0-P3) from the following mice strains C57Bl/6J (wild type), PI3K$\gamma^{-/-}$ (knock-out) and PI3K$\gamma^{KD/KD}$ (knock-in). The hippocampus has been removed and the brains from always two neonatal mice have been collected in a Falcon tube (15mL) together with the autoclaved PBS. PBS has been replaced with 2 mL dissociation medium (containing 20 mM HEPES, 0,05% Trypsin, 700 µM EDTA, and 12 µg/mL DNase in DMEM) and incubated for 30 min at 37°C. Afterwards the mixed culture has been homogenized, added into a T75 Cell Culture flask with culture medium - DMEM complete (containing additionally 10% FCS heat-inactivated, 100 U/mL Penicillin, 100 µg/mL Streptomycin, 2,5 µg/mL Amphotericin B) and incubated for 14 days in the incubator (37 °C, 5% CO_2). The culture medium has been changed once a week.

Isolation of primary microglial cells have been done by using 2 mM EDTA (in autoclaved PBS) and gently shaking the flask so the microglial cells which are on the top have been collected in culture medium and astrocytes remain confluent on the bottom of the flask. Purity of microglia was always in the range between 95-98%, as confirmed by Iba1 staining (data not shown). All experiments were carried out in accordance with the German legislation on protection of animals and with permission of the local animal welfare committee (TWZ07-2017).

3.2.2. Stimulation – "two hit" approach

In order to induce adaptive (memory-like) response, microglial cells (75.000 cells/well) were seeded in a 12-well plate and were stimulated twice following a two-step ("two hit") approach, consisting of initial priming with different doses of LPS (1 fg/mL – 100 ng/mL) or β-glucan (1 fg/mL – 1 µg/mL) for 24h as described in Figure 3. Thereafter cells have been washed up and the medium has been changed. On day 6, microglial cells were re-stimulated with a fixed dose of LPS (100 ng/mL) or β-glucan (1 µg/mL). In parallel, we performed also control groups such as unstimulated (US) - without any stimulation, and unprimed (UP) - stimulated only on day 6 by

LPS or β-glucan. Samples have been collected in a period of 6h (RNA samples) and 24h (lysates and supernatants) after the 2nd stimulation.

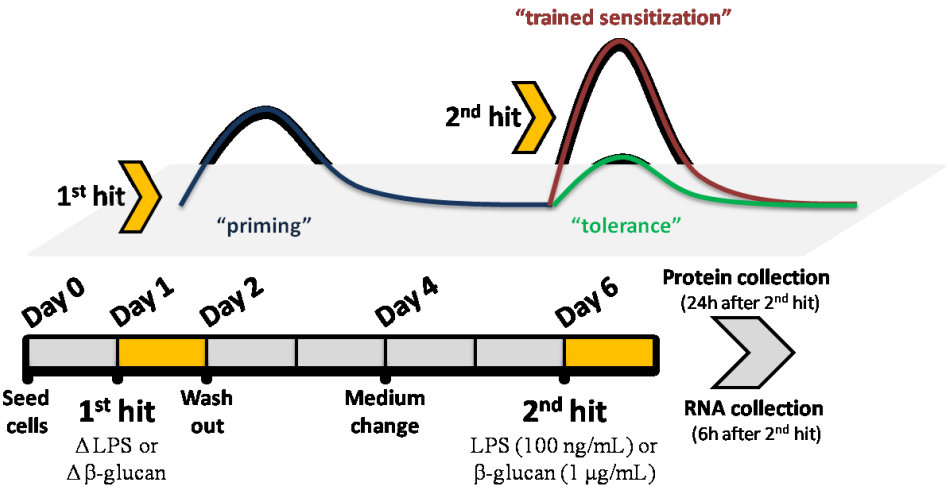

Figure 3: Schematic view of the "two hit" approach to induce different adaptive response.
Primary microglial cells (75.000 cells/well) were seeded on day 0 and stimulated with different doses of LPS (1 fg/mL – 100 ng/mL) or β-glucan (1 fg/mL – 1 µg/mL) on day 1 for 24h. On day 2 cells were washed up and followed by medium change on day 4. Thereafter cells were left to rest and on the day 6 have been re-stimulated with a fixed dose of LPS (100 ng/mL) or β-glucan (1 µg/mL). 6h and 24h after the 2nd stimulation samples have been collected and processed for further analyses.

3.2.3. Enzyme-linked immunosorbent assay (ELISA)

Cell culture media were assembled 24h after the second stimulation and centrifuged at 10.000 x g for 5 min at 4°C. Afterwards, the supernatants of each sample has been collected and immediately stored at -80°C for later to measure the cytokine production. Sandwich enzyme-linked immunosorbent assay (ELISA) has been used to determine the levels of different pro-inflammatory (TNF-α, IL-6) and anti-inflammatory (IL-10) cytokines. Different kits from BioLegend were used for this issue following the manufacturer instructions to run the assay (Table 2). First, 96 well Nunc-Immuno plates have been coated with coating buffer (8,4 g $NaHCO_3$, 3,56 g Na_2CO_3, pH to 9,5) containing Capture Antibody (ratio 1:200) 1 day prior to running ELISA (at 4°C). 24h after the incubation time, plates have been washed thrice with 300 µL/well wash buffer (PBS + 0,05% Tween-20) and incubated for 1h with 200 µL/well Assay Diluent (shaking) in order to block the non-specific binding and reduce the background. During this incubation time, standards and samples have been prepared and diluted using the assay diluent (1% BSA, filtered – 0,2 µm diameter) as required. Afterwards the plate has been washed

four times, followed by adding 100 µL/well of each standard and samples as duplicate in the appropriate wells and incubated for 2h (shaking). Again four times washing, and incubated with Detection antibody (100 µL/well, 1:200) in assay diluent for 1 hour. Then the plates have been washed again, followed by adding the enzyme Avidin-HRP (100 µL/well, 1:1000) diluted in assay diluent and incubated for 30 minutes with shaking (in the dark). After 30 minutes the plates have been washed five times and incubated (in the dark) with 100 µL/well TMB Substrate Solution until the desired blue color has been developed (15 - 30 minutes). In the end, the reaction (development of the color) was interrupted with stop solution by changing the pH and thus inactivating the enzyme (Avidin-HRP). 2N H_2SO_4 or 0,12N (1%) HCl are recommended for this issue. The absorbance was read at 450 nm with a second reference wavelength at 570 nm in VersaMax Microplate Reader (Molecular Devices, USA).

Cytokine levels of TNF-α, IL-6 and IL-10 were normalized to the protein concentrations of each sample.

3.2.4. SDS page and Western blotting

Protein expression was analyzed by Western blotting of cell lysates 24h after the second stimulation. Briefly, cells were lysed using ice cold RIPA buffer (50 mM Tris-ultrapure, 150 mM NaCl, 1% NP-40, 0,5% Sodium deoxycholate, 0,1% SDS, pH adjusted to 8,0) to extract proteins from the cell culture samples. Prior to use, RIPA buffer has been supplemented freshly with the protease and phosphatase inhibitor cocktail (1 mg/mL Leupeptin ratio 1:500, 1 mg/mL Pepstatin A ratio 1:1000, 10 mM Na-orthovanadate ratio 1:500, 100 mg/mL Pefabloc ratio 1:1000) in order to prevent proteolytic degradation during cell lysis and protein extraction. Samples were centrifuged (13.500 x g for 30 min at 4°C), and supernatants were mixed with 5 x protein sample buffer - Lämmli buffer (33% Glycerol, 5% SDS, 25% 2-Mercaptoethanol, 0,1 mg/mL of 1% Bromophenol blue, 83 mM Tris, pH adjusted to 6,8) and heated for 5 min at 95°C.

For the collection and separation gels the collection (0,5 M Tris-HCl, pH to 6,8) and separation buffers (2 M Tris-HCl, pH to 8,8) were used combined with 30% Acrylamide/bis-acrylamide, 20% SDS, 20% APS and TEMED. Proteins were separated in 10% SDS- polyacrylamide gel electrophoresis (SDS-PAGE) buffer (25 mM Tris, 0,2 mM Glycine, 3,5 mM SDS) with a

constant 120 volt (V) and 30 milliampere (mA) per gel. Proteins have been transferred using Transfer buffer (48 mM Tris, 39 mM Glycine, 1,3 mM SDS, 15% Methanol) from the gel to a 0,45 µm polyvinylidenfluoride (PVDF) membrane by a Semi-Dry-Blotting Machine (Millipore Graphite, Millipore USA) with a constant current (1 mA/cm^2, 1h). Thereafter, the membrane was blocked with 1% BSA in TBS-T (50 mM NaCl, 0,1% Tween 20, 5 mM Tris-HCl, pH to 7,4) for 30 min at room temperature (with shaking). Then the membrane was immunoblotted overnight at 4°C (shaking) with primary antibodies mentioned above (Table 3), washed thrice with TBS-T (10 - 15 min/wash) and incubated with HRP-conjugated secondary antibody (1:10 000 in 1% BSA or 5% milk powder) for 1h at room temperature with shaking. Afterwards the membrane has been washed three times (5 min/wash) with TBS-T, and flushed with Western Lightning Plus-ECL Enhanced Chemiluminescent Substrate.

Protein bands were detected by enhanced chemiluminescence reaction using LAS4000 camera (Fuji Photo Film Co., Tokyo, Japan). Quantification of the protein bands on the membrane was done using the FujiFilm Multi Gauge Ver. 3.0 software (Fuji Photo Film Co., Tokyo, Japan).

3.2.5. Protein quantification

Total protein concentration was determined using the Pierce™ 660 nm Protein Assay Kit (#22662) from Thermo Fisher Scientific (Massachusetts, USA). Ionic detergent compatibility reagent (IDCR) (#22663, Thermo Fischer Scientific) has been added to the Protein Assay Reagent prior to use in order to increase the detergent compatibility and reduce the interference. For the assay 10 µL of each replicate sample, standard and blank were added into a 96 well/plate, followed by 150 µL of the above mentioned protein assay reagent (containing IDCR) per well and incubated for 1 minute with shaking. Furthermore the plate has been incubated at room temperature for 5 minutes without shaking it. Then, absorbance was measured at 660 nm using TECAN Infinite 200 Plate reader (Tecan, Switzerland). Protein concentration is then calculated based on the values of the standard curve.

3.2.6. Seahorse assay and crystal violet staining

Metabolic flux analysis was performed using a Seahorse XF96 Extracellular Flux Analyzer (Agilent technologies). 20.000 primary microglial cells were seeded on 96-well Seahorse XF96 microplates. Cells were treated as described above ("two hit" approach). Before the experiment, supernatant medium was replaced with Seahorse XF Assay Medium (Agilent Technologies, pH adjusted to 7,4), supplemented with 10 mM D-glucose and 1 mM sodium pyruvate. Cells were then incubated for additional 1h in a CO_2-free incubator at 37°C. Basal oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured before adding 2 µM oligomycin (Abcam), 30 µM 2,4-dinitrophenol and 2 µM antimycin A (Sigma). Each compound was injected consecutively. OCR and ECAR were measured as pmoles/min and mpH/min respectively, in cycles of 3 min mix and 3 min measure periods at 37°C. At the end of the measurement cells densities per well were quantified by crystal violet staining. The observed OCR and ECAR were normalized to corresponding cell densities. Wave software (Agilent Technologies) was used to analyze the data sets. After fixation for 10 min in 100% methanol at room temperature (RT), cells were incubated for 30 min at 37°C in a 0.05% crystal violet solution (in H_2O). Following, cells were washed two times with H_2O and cell bound crystal violet was resolved in 10% acetic acid whilst shaking on an orbital shaker. The resulting absorption was measured on an Infinite® M1000 PRO microplate reader (Tecan).

3.2.7. Measurement of reactive oxygen species (ROS)

Reactive oxygen species were measured using the H_2DCFDA-assay. The assay is based on the use of 2', 7'-Dichlordihydrofluorescein-diacetat (H_2DCFDA; #D399, Thermo Fisher Scientific, Waltham, MA, USA), a membrane-permeable reduced form of fluorescein which reacts with reactive oxygen species, and then turning fluorescent. Therefore, microglial cells were seeded into white clear bottom 96-well plates (30.000 cells/ well). After becoming adherent, cells were stimulated twice with LPS, as described above. 24h after the second stimulation the medium was aspirated and 200 µl of H_2DCFDA-solution (stock 50 mM 1:1000 in 10 mM HEPES/$CaCl_2$) was added and cells were incubated for 20 min at 37°C. Thereafter, cells were carefully washed twice with an HEPES/$CaCl_2$ solution. Measurement of intracellular ROS levels was performed at 485

nm excitation and 535 nm emission using TECAN Infinite 200 Plate reader (Tecan, Switzerland).

3.2.8. Lactate production measurements

Supernatants from microglial culture were used to measure lactate production by sequential enzymatic reactions, according to (Lin et al. 1999). First, lactate oxidase (LO; #L0638, Sigma-Aldrich) converts lactate and oxygen to pyruvate and H_2O_2. In the second reaction, catalyzed by the horseradish peroxidase (HRP; #77332, Sigma-Aldrich), in the presence of H_2O_2, the chromogenic substrate ABTS (#A1888, Sigma-Aldrich) produces a colored dye which can be measured with a spectrophotometer at 405 nm. Lactate concentration has been calculated from a standard curve of known concentration of lactate (Lactate Standard for IC – TraceCERT; #07096, Sigma-Aldrich). Lactate levels were normalized to the protein concentrations of each sample.

3.2.9. cAMP measurements

Primary microglial cells (5.000 cells/well) have been seeded in 96 well/plates (clear-bottom) and treated as described above ("two hit" protocol). 24h after the 2nd stimulation, cyclic AMP production has been measured using cAMP GloAssayKit (#V1501, Promega) following manufactory's protocol. Measurement of the luminescence was performed using TECAN Infinite 200 Plate reader (Tecan, Switzerland), and the cAMP values have been calculated based on the values of the standard curve.

3.2.10. *In vitro* phagocytosis assay

Phagocytic efficiency has been investigated as described (Sun et al. 2008). Microglial cells (75.000 cells/ well) were seeded on cover slips in 12 well/plates and treated as described above (Fig. 3). 2 µL FITC labeled Zymosan A (S. cerevisiae) Bio Particles (9800U/mL) (#Z2841, Thermo Fischer Scientific) per 75.000 cells have been used to perform the phagocytosis assay.

After 1h incubation with Zymosan A at 37°C (5% CO_2) cells were fixed with 500 µL/well Formalin 5%, washed three times with 2% NDS (in 1 x PBS) and blocked with 500 µL/well 10% NDS in PBS-Tween 0.1%. Cells were first incubated with 300 µL/well Iba1 primary antibody (goat Iba1, #ab5076, Abcam) for 1h at room-temperature, thereafter washed with 1 x PBS and incubated with 300 µL/well secondary antibody (Alexa Fluor® 568 donkey anti goat IgG). Cells then were stained with DAPI-solution for 10 min (2 µg/mL, ratio 1:1000 in 1 x PBS) and washed four times with PBS. Cover slips (24 x 32 mm) then were mounted with Fluoromount-G (#0100-01, Southern Biotech) into microscope slides. Phagocytosed particles and cells of five independent visual fields were counted under a fluorescence microscope. The result of the phagocytosis was calculated by determining the phagocytic index (the uptake rate of FITC-Zymosan particles per cell).

3.2.11. *In vivo* phagocytosis assay

Experiments were performed on adult (10–14 weeks) WT, PI3K$\gamma^{-/-}$ and PI3K$\gamma^{KD/KD}$ mice (7 mice per group) kept at neutral ambient temperature (T=30°C) during the whole experimental period. According to the "two hit" approach, mice were initially injected with either low dose LPS (0.025 mg/kg, i.p.) or high dose LPS (10 mg/kg, i.p.) for priming and 3 days later with a subsequent application of 10 mg/kg, i.p. LPS. Administration of fluorescein isothiocyanate (FITC)-labeled Zymosan particles (9800U/mL) were performed 24 hours after the second LPS injection as described previously (Schmidt et al. 2013). Mice were anesthetized by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (16 mg/kg), and were positioned in a stereotaxic apparatus (Stoelting, Wood Dale, IL, USA). Mice were then placed on a homoeothermic heat blanket to maintain normal body temperature during surgery. The skull was exposed by a skin incision, and small burr holes were drilled through the skull. Using a micromanipulator cannula (diameter 0,24 mm) attached on a Hamilton micro syringe (0,5 µL) was stereotaxically placed into the thalamus on both sides (stereotaxic coordinates were AP: 2,0 mm; L: ± 1,5 mm; and V: 2,0 mm). Subsequently, 20 nL of FITC-labeled Zymosan particles suspended in artificial cerebrospinal fluid were infused within 120 sec. The cannula remained in place for 5 min before removal. 24h later mice were deeply anesthetized and perfused with 4% paraformaldehyde (PFA) in phosphate buffer by cardiac puncture via the left ventricle.

Brains were removed immediately after fixation and post-fixed for 5h in 4% PFA at 4°C. After cryoprotection in PBS containing 30% sucrose, brains were frozen in methyl butane at -30°C and stored at -80°C. Whole brains were cut by coronal sections at 40 µm on a freezing microtome (Microm International GmbH, Thermo Scientific, Germany). The slices were immuno-stained with anti-Iba1 antibody to visualize microglia. An altitude of 40 µm was predefined as region of interest. Z-stack imaging was performed with a 20x objective using fluorescence microscope (Nikon Eclipse Ti, Nikon Instruments - Japan). Quantitative measurements were done using ImageJ software (NIH, Bethesda, MD), blinded to the treatment groups were used to count the percentage number of Iba-1 positive cells per voxel mm^3 containing Zymosan particles.

3.2.12. Blood plasma and brain tissue cytokine measurements

Cytokines levels of TNF-α and IL-6 in blood plasma and brain tissue were determined following the manufacturer instructions using BioLegend kits as shown previously (Table 2). Briefly, blood samples were collected in heparin coated tubes (1,5 mL) by the heparinized needles and centrifuged for 10 min at room temperature at 2.000 x g, followed by collecting in the new tubes the blood plasma and stored at -80°C. The brain tissue was harvested after rinsing with cold PBS, immediately put in ice cold methyl butane and kept at -80°C until processing. The brain tissue was then powdered in homogenization buffer (50 mM Tris, 150 mM NaCl, 5 mM EDTA; pH to 8,0) containing phosphatase and protease inhibitors, and then centrifuged at 10.000 x g for 10 min at 4°C and supernatant were stored at -80°C until the measurement. Cytokine production data were normalized to the protein concentrations of each sample.

3.2.13. RNA isolation and real-time PCR

6 hours after the 2^{nd} hit, media has been removed and further preceded to RNA isolation. To determine gene expression levels, total RNA was extracted using 0,5 mL QIAzol Lysis Reagent (#79306) purchased from Qiagen (Hilden, Germany), followed by 0,2 mL chloroform into 2 mL tubes and centrifuged at 12.000 x g for 15 min at 4°C. After the centrifugation, the colorless upper phase was collected in new 1,5 mL tubes and further proceeded to the RNA precipitation

procedure by using 0,5 mL of 100% isopropanol (propanol-2) (incubated at RT for 10 min). Samples were centrifuged at 12.000 x g for 10 min at 4°C and processed to the RNA wash step, where the upper phase has been removed leaving only the RNA pellet. The pellet was washed with 1 mL 75% ethanol, centrifuged again (7.500 x g, 5 min at 4°C) and left for 10 - 15 minutes to air dry the pellet. Then the pellet was dissolved in 40 µL RNase - free water (PCR water) and stored at -80°C. RNase Away solution was used to flush pipettes and other equipments in order to prevent any contamination with other RNases or DNAs. RNA concentration and quality were checked using the Nanodrop ND-1000 machine (Peqlab, Erlangen, Germany). Complementary DNA (cDNA) was synthesized using RevertAid First Strand cDNA Synthesis kit (#K1612) from Thermo Fisher Scientific (Waltham, MA, USA). Real-time qPCR reaction was performed using Realplex Mastercycler EpGradient S (Eppendorf AG, Germany). Primers were prepared using 1 x TE buffer and used in a concentration of 10 µM as depicted in Table 6. GAPDH was used as housekeeping gene for normalization of the target genes. Relative gene expression was calculated using the comparative C_T ($2^{-\Delta\Delta C_T}$) method (Schmittgen and Livak 2008).

Table 6: Primers used for real-time PCR

Genes	Forward primer (5'-3')	Reverse primer (5'-3')
GAPDH	CATGGCCTTCCGTGTTTCCTA	CCTGCTTCACCACCTTCTTGAT
HIF-1α	CTCATCAGTTGCCACTTCC	TCATCTTCACTGTCTAGACCAC
IL-10	ACCAGCTGGACAACATACTGC	TCACTCTTCACCTGCTCCACT
IL-6	CCTCTCTGCAAGAGACTTCCATCCA	GGCCGTGGTTGTCACCAGCA
iNOS	AAGGCCACATCGGATTTCAC	GATGGACCCCAAGCAATACTT
MyD88	TCCGGCAACTAGAACAGACAGACT	GCGGCGACACCTTTTCTCAAT
TLR4	ACCTGGCTGGTTTACACGTC	CAGGCTGTTTGTTCCCAAAT
PFKFB3	GGAGAGGTCAGAGGATGCAAA	GCTGTTGATGCGAGGCTTTT
TNF-α	CTGTAGCCCACGTCGTAGC	TTGAGATCCATGCCGTTG
TRIF	CACAGTCCCAATCCTTTC	TCACTCTGGAGTCTCAAG

3.2.14. Statistical analysis

Graphs were prepared using GraphPad Prism6 software (GraphPad Software, La Jolla, CA, USA), and statistical analysis were carried out using SigmaPlot Software (SigmaPlot Software, San Jose, USA). Data are presented as box plots showing the medians with the boxes ranging from 25^{th} -75^{th} percentile and whiskers ranking form 10^{th} to 90^{th} percentiles (extreme data are marked outside). Comparisons between groups were made with one or two-way analysis of variance (ANOVA), followed by Holm-Sidak post-hoc test. Differences were considered significant when $P<0.05$, where * indicates differences vs. unprimed state, and § indicates differences vs. cells obtained from wild-type mice.

Grubbs' test has been used to remove extreme outliners in order to show reasonable data analysis.

IV. Results

4.1. LPS and β-glucan induce dose-dependent adaptive responses in primary microglial cells

4.1.1. Paradigmatic analysis of priming effects resulting from incremental increase of stressor concentration

The primary aim of this project was to investigate the ability of the microglia to induce trained sensitization and tolerance after subsequent challenge with different stressors. Initially, single stimulations with different doses of LPS, starting from 1 fg/mL up to 100 ng/mL LPS, affected the production of pro-inflammatory cytokines (TNF-α, IL-6) in a dose-dependent manner, where increasing doses of LPS (>100 pg/mL) significantly enhanced the levels of cytokine release in primary microglial cells (Fig. 4).

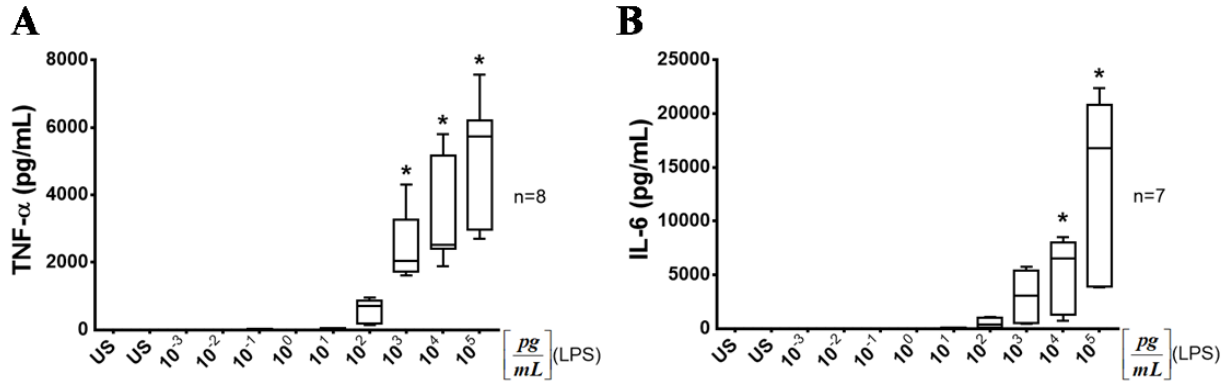

Figure 4: Production levels of TNF-α and IL-6 after single stimulation with different doses of LPS. Primary microglial cells were stimulated with different doses of LPS, starting from 1 fg/mL – 100 ng/mL for 24 hours. Supernatants were collected and the cytokine levels for: A) TNF-α, B) IL-6 have been assayed by ELISA. Data are shown as boxplots and whiskers, *p <0.05, * vs. unstimulated condition, 1-way-ANOVA (Holm-Sidak post-test).

In order to induce adaptive responses, a so called "two hit" approach was used as described above (see 3.2.2.). Gene expressions and protein expression of iNOS as well as TNF-α release were assayed by real-time PCR, western blotting and ELISA. Results show that low doses primed primary microglial cells, especially at 1 fg/mL LPS show significantly increased levels of TNF-α known as trained sensitization, whereas cells primed with higher doses of LPS exhibited

tolerance behavior exemplified by decreased levels of TNF-α compared to the unprimed state (Fig. 5A-C, E).

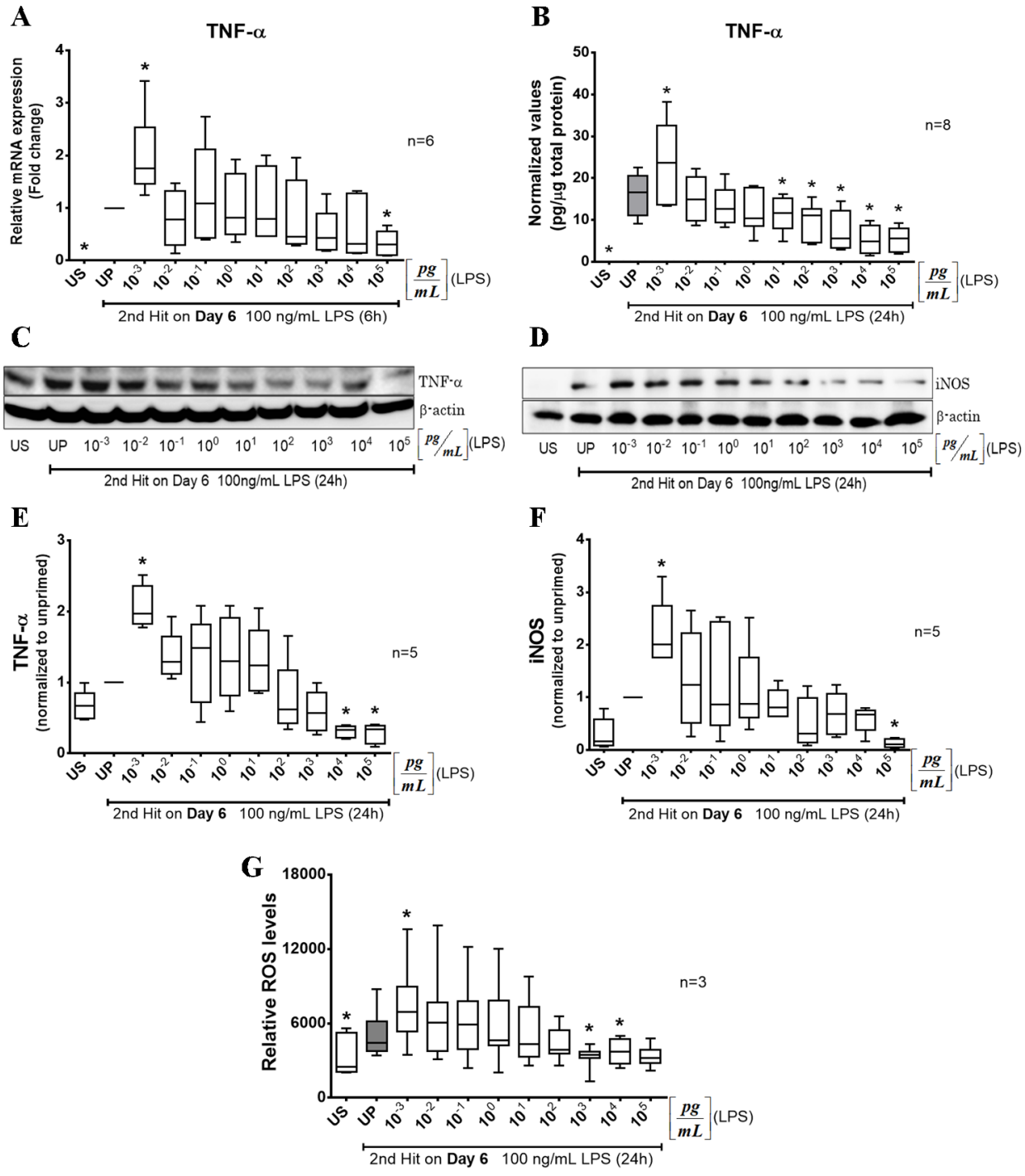

Figure 5: LPS induces trained sensitization and tolerance in a dose-dependent manner in primary microglial cells primed with different doses of LPS after subsequent re-stimulation.

Microglial cells have been primed with increasing doses of LPS (1 fg/mL – 100 ng/mL) on day 1 and re-stimulated with 100 ng/mL LPS on day 6. RNA samples (6h), lysates and supernatants (24h) have been collected after the 2nd stimulation and analyzed for TNF-α by: A) real-time PCR (normalized to GAPDH and are relative to unprimed state (assigned as 1.0)), B) ELISA immuno-assay (normalized to total protein concentration) and C) Western blot and quantified values (E). iNOS protein expression and quantification have been assayed by Western blotting (D, F), whereas ROS was assayed using H_2DCFDA-oxidation (G). Data are shown as boxplots and whiskers, *p <0.05, * vs. unprimed condition (grey column), 2-way-ANOVA (Holm-Sidak post-test). US, unstimulated; UP, unprimed.

We further investigated the protein expression of inducible NO synthase (iNOS) as inflammatory mediator, important for NO production in microglial cells. As shown in Fig. 5D, F, iNOS expression was significantly increased in microglial cells primed with 1 fg/mL LPS indicating trained sensitization, whereas increasing doses, especially at 100 ng/mL LPS induced a strong reduction of the iNOS expression indicating trained tolerance. In addition, ROS production was similarly altered, e.g. an enhanced ROS production has been verified in microglial cells primed with low-dose LPS as well as diminished levels after priming with higher LPS doses (Fig. 5G).

In order to verify PAMP species-independent priming effects, the fungal PAMP β-glucan was used. As shown in Fig. 6A microglial cells have been stimulated with increasing doses, from 1 fg/mL up to 1 μg/mL of β-glucan. Data show that β-glucan did not provoke any changes in the TNF-α production with increasing doses, except in the highest β-glucan dose used. In addition, in order to answer the question if β-glucan priming of primary microglial cells *in vitro* can lead to similar dose-dependent adaptive responses as priming with LPS, we re-challenged the cells with a fixed concentration of LPS (100 ng/mL). As shown in Fig. 6B, microglial cells primed with 0.1 pg/ml β-glucan exhibited trained sensitization characterized by increased TNF-α levels compared to the unprimed state, whereas doses equal or higher than 100 pg/mL, exclusively the highest dose of β-glucan (1 μg/mL) lead to decreased production of TNF-α in microglial cells.

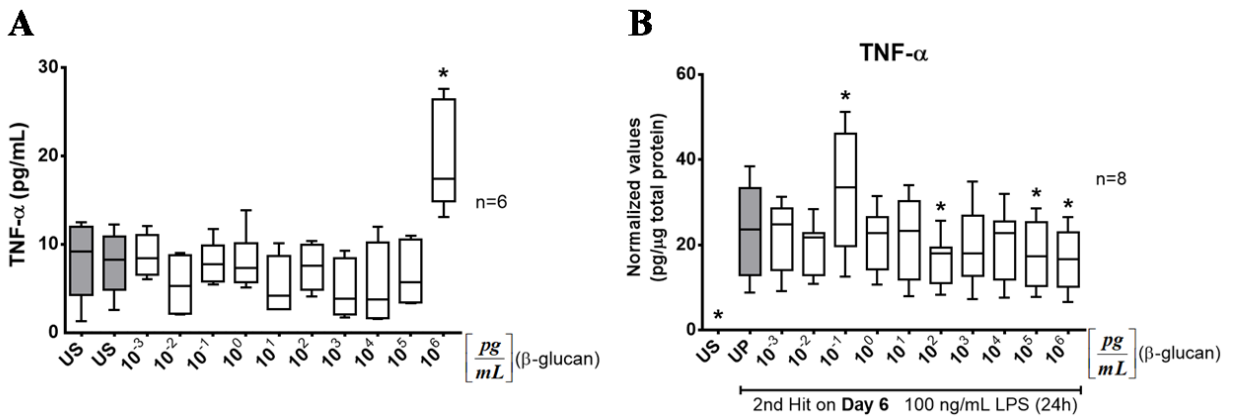

Figure 6: β-glucan induces dose-dependent adaptive responses, trained sensitization at low doses and tolerance in higher concentrations, after re-challenge with LPS.
(A) Single dose response with β-glucan shows no significant alterations in TNF-α production, expect 1 µg/mL which shows increasing amounts. Microglial cells stimulated using the "two hit" approach, primed with different doses of β-glucan, are capable to induce increasing levels of TNF-α (B) at 100 fg/mL, whereas higher doses, notably 1 µg/mL suppress the production of TNF-α. Data are shown as boxplots and whiskers, *p <0.05, * vs. unstimulated condition (grey columns) (A), 1-way-ANOVA (Holm-Sidak post-test); and *p <0.05, * vs unprimed condition (grey column) (B), 2-way-ANOVA (Holm-Sidak post-test). US, unstimulated; UP, unprimed.

As shown above, microglial cells primed with β-glucan were re-challenged with 100 ng/mL LPS, raising the question if β-glucan (1 µg/mL) re-stimulation can induce similar adaptive responses. Microglial cells were primed with β-glucan and re-challenged again with β-glucan (1 µg/mL).

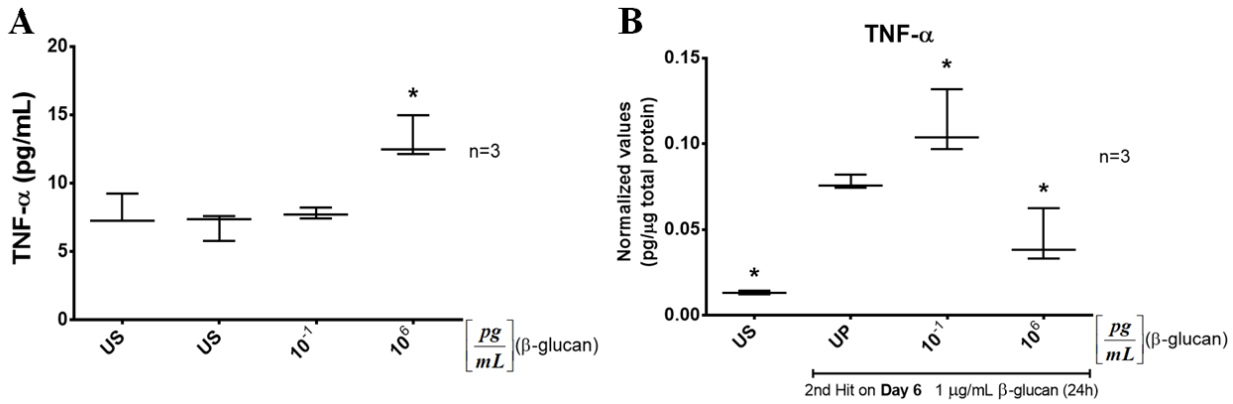

Figure 7: TNF-α production after subsequent stimulation with the same stressor, β-glucan.
Single stimulation triggers enhanced production of TNF-α at 1 µg/mL (A), re-challenge of the microglial cells with β-glucan leads to similar adaptive responses, with enhanced levels at 100 fg/mL and decreased levels at 1 µg/mL (B) of TNF-α measured by ELISA immuno-assay. Data are shown as boxplots and whiskers, (A) *p <0.05, * vs. unstimulated condition, 1-way-ANOVA (Holm-Sidak post-test); and (B) *p<0.05, * vs. unprimed condition, 2-way-ANOVA (Holm-Sidak post-test). US, unstimulated; UP, unprimed.

Results showed that priming with 100 fg/mL β-glucan induces increased levels of TNF-α, whereas the concentration of 1 µg/mL reduces significantly the production of TNF-α after subsequent challenge (Fig. 7). Altogether our data indicate that different PAMPs (LPS and β-glucan) are able to induce trained sensitization when primed with very low PAMPs doses, whereas microglial cells primed with high PAMPs doses lead to reduced levels of the inflammatory mediators indicating tolerance behavior.

4.1.2. Identification of priming effects upon various pro- and anti-inflammatory responses in microglial cells

Based on the data resulting from incremental increase of stressor concentrations presented in 4.1.1., we decided to focus further studies on trained sensitization and tolerance using stressor concentrations with maximum priming effects (trained sensitization: LPS: 10^{-3} pg/mL, β-glucan: 10^{-1} pg/mL; trained tolerance: LPS: 10^{5} pg/mL, β-glucan: 10^{6} pg/mL).

First, additional indicators for pro-inflammatory microglial response were studied. As shown in Fig. 8, overall microglial response after priming and re-stimulation regarding IL-6 production was similar as for TNF-α production, e.g. microglial cells primed with 1 fg/mL LPS exhibited trained sensitization after subsequent challenge, whereas cells primed with 100 ng/mL showed after re-stimulation reduced levels of IL-6 known as tolerance response.

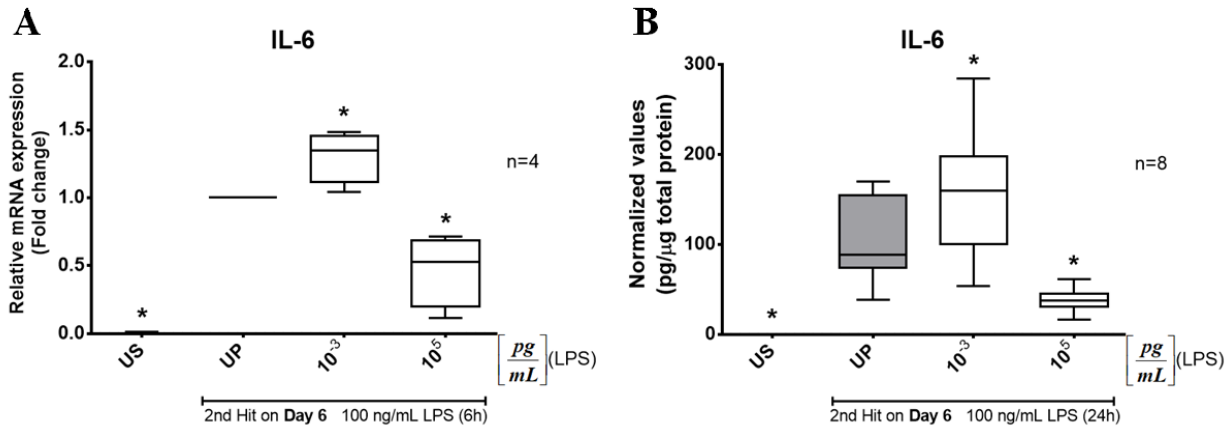

Figure 8: IL-6 production in primary microglial cells after priming and subsequent stimulation by LPS.

Microglial cells have been primed with two different doses of LPS (1 fg/mL and 100 ng/mL) on day 1 and re-stimulated with 100 ng/mL LPS on day 6. RNA samples (6h) and supernatants (24h) have been collected after the 2nd hit and analyzed for IL-6 using: A) real-time PCR (normalized to GAPDH and are relative to unprimed state (assigned as 1.0)), and B) ELISA immuno-assay (normalized to total protein concentration). Data are shown as boxplots and whiskers, *p <0.05, * vs. unprimed condition (grey column), 2-way-ANOVA (Holm-Sidak post-test). US, unstimulated; UP, unprimed.

Furthermore, microglial cells primed with β-glucan displayed similar adaptive dose-response, e.g. an increased production of IL-6 by priming with 100 fg/mL β-glucan and decreased levels of IL-6 by priming with 1 µg/mL β-glucan (Fig. 9).

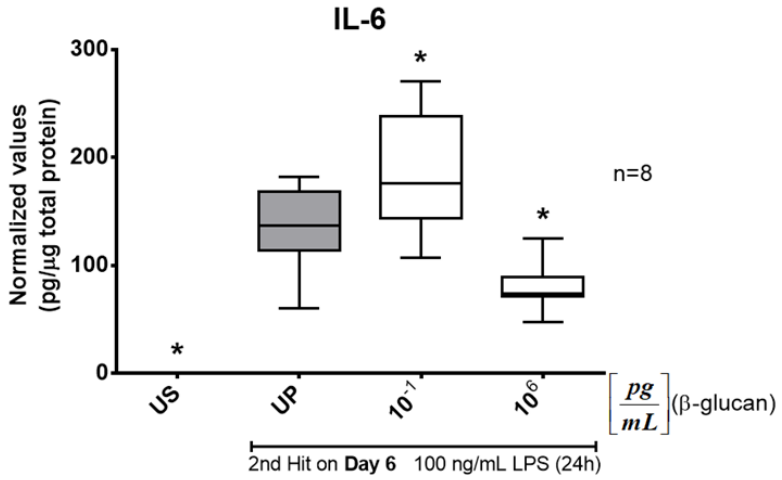

Figure 9: IL-6 production amounts of microglial cells after priming with different doses of β-glucan and subsequent stimulation by LPS.
Microglial cells stimulated using the "two hit" approach, primed with different doses of β-glucan (100 fg/mL and 1 µg/mL), induce increasing levels of IL-6 at 100 fg/mL, whereas 1 µg/mL suppresses the production of IL-6. Data are shown as boxplots and whiskers, *p <0.05, * vs. unstimulated condition (grey column), 2-way-ANOVA (Holm-Sidak post-test). US, unstimulated; UP, unprimed.

In order to confirm the above findings about iNOS protein levels, we analyzed the messenger RNA (mRNA) expression of iNOS and found also that after priming with 1 fg/mL LPS the iNOS expression was up-regulated, whereas priming with 100 ng/mL LPS led to a down-regulation in primed microglial cells after subsequent LPS challenge (Fig. 10A). A similar response pattern was shown for gene expression of HIF-1α (Fig. 10B).

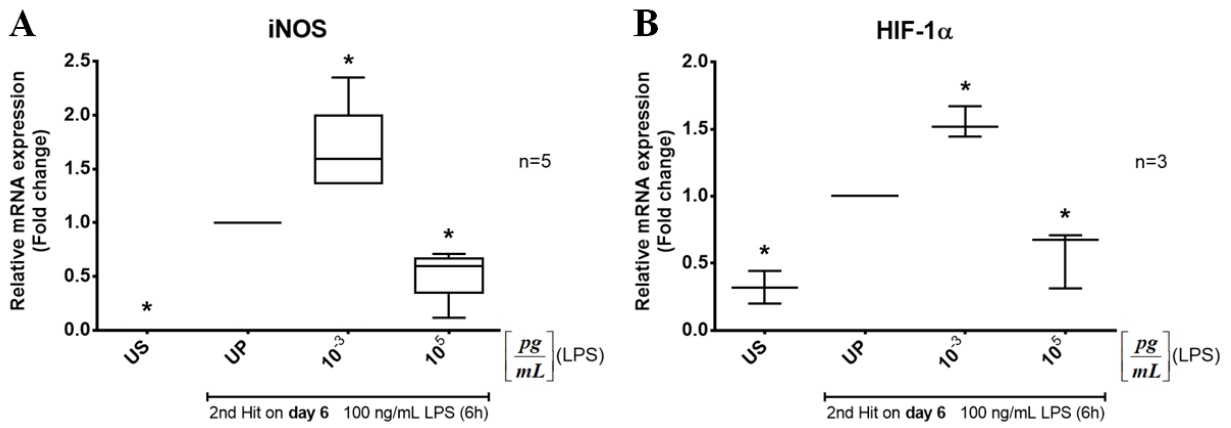

Figure 10: Increased mRNA levels of iNOS and HIF-1α indicating trained sensitization after low-dose priming of microglial cells and down-regulation indicating tolerance response in microglial cells after high-dose priming with LPS.
Gene expression was analyzed by real-time PCR using cDNA samples of the targeted RNA samples: A) iNOS and B) HIF-1α. Cells have been stimulated using a "two hit" approach: priming with different doses (1 fg/mL – 100 ng/mL) and on day 6 re-challenging them with a fixed dose (100 ng/mL) LPS. Data are shown as boxplots and whiskers, *p <0.05, * vs. unprimed condition, 2-way-ANOVA (Holm-Sidak post-test). US, unstimulated; UP, unprimed.

Therefore, microglial cells are able to induce trained sensitization as response to low-dose priming with LPS, mainly characterized with increased expression of different pro-inflammatory mediators similar to a "M1"-like phenotype of microglia. Moreover, cells after high-dose priming develop tolerance behavior characterized with decreased levels of pro-inflammatory mediators.

To further investigate additional features of tolerance behavior of microglial cells, we evaluated also the expression and production of the anti-inflammatory mediators such as Arg-1, IL-10, IL-4, and TGF-β. As anticipated, high-dose primed microglial cells that induce a tolerant response, displayed increased levels of the anti-inflammatory mediators at the gene regulatory as well as protein expression level, corresponding to the so called "M2"-like phenotype (Fig. 11).

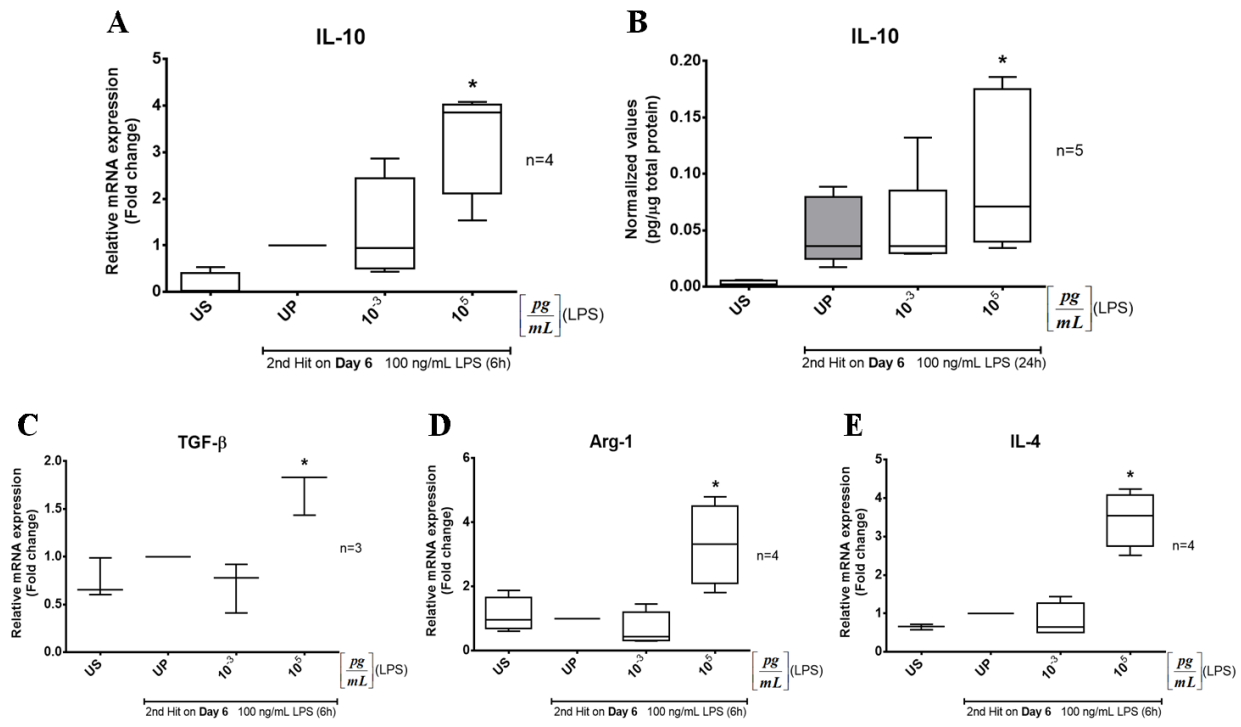

Figure 11: Increased anti-inflammatory markers during tolerance in primary microglial cells.
Primary cells have been stimulated using the "two hit" approach. Priming with 100 ng/mL LPS triggers increased expression of IL-10 (A), TGF-β (C), Arg-1 (D) and IL-4 (E) measured by real-time PCR, data are normalized to GAPDH representing relative values to unprimed state (assigned as 1.0). Moreover the production of the anti-inflammatory cytokine IL-10 has been enhanced as well in the primary microglial cells pre-stimulated with 100 ng/mL LPS, assayed by ELISA immuno-assay and normalized to protein levels. Data are shown as boxplots and whiskers, *p <0.05, * vs. unprimed condition (grey column), 2-way-ANOVA (Holm-Sidak post-test). US, unstimulated; UP, unprimed.

Since trained sensitization and tolerance responses are characterized also by different metabolic states, we investigated the lactate production in primary microglial cells, as an indirect parameter to verify the glycolytic activity of cells. Our data showed that low-dose primed microglial cells exhibited an increased lactate production, whereas high-dose primed microglial cells showed decreased levels of lactate (Fig. 12).

In addition we analyzed the gene expression of the 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3), a gene associated with glycolysis and found that there was a partly similar response as shown for lactate production, e.g. an enhanced expression after low-dose priming (Fig. 12).

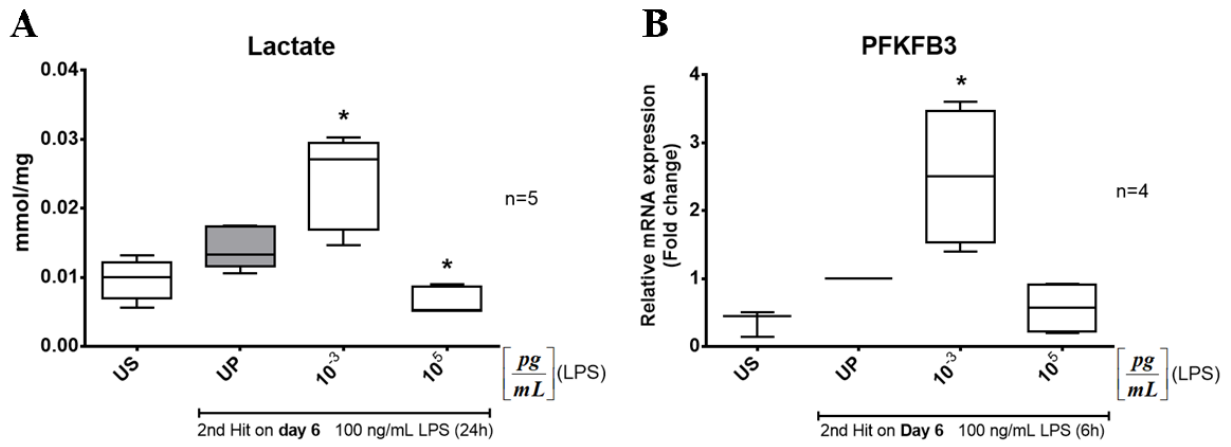

Figure 12: Increasing glycolysis during trained sensitization showed by increased lactate production and PFKFB3 expression.
The "two-hit" approach has been used to stimulate primary microglial cells. Lactate production (A) has been measured by sequential enzymatic reactions (see 3.2.8.). PFKFB3 expression has been assayed using real-time PCR and the data are normalized to GAPDH representing relative values to unprimed state (assigned as 1.0). Data are shown as boxplots and whiskers, *p <0.05, * vs. unprimed condition (grey column), 2-way-ANOVA (Holm-Sidak post-test). US, unstimulated; UP, unprimed.

Therefore, primary microglial cells *in vitro* are capable to respond with opposed adaptive patterns, trained sensitization with low-dose priming and tolerance (persistence) behavior owing to high-dose priming with different PAMPs.

4.2. PI3Kγ regulates cytokine production during trained sensitization and tolerance

Considering the above findings that microglia are capable to induce adaptive responses in a dose-dependent manner- trained sensitization at 1 fg/mL and tolerance at 100 ng/mL LPS, in addition to our group data about PI3Kγ, we hypothesized that PI3Kγ has a crucial role mediating trained sensitization and tolerance in microglial cells. Since one of the key characteristics of the trained sensitization was increased production of pro-inflammatory cytokines, whereas during tolerance that inflammatory response was significantly decreased, in addition to increased anti-inflammatory activity, we decided to investigate the role of PI3Kγ controlling the cytokine profile in primary microglial cells *in vitro*.

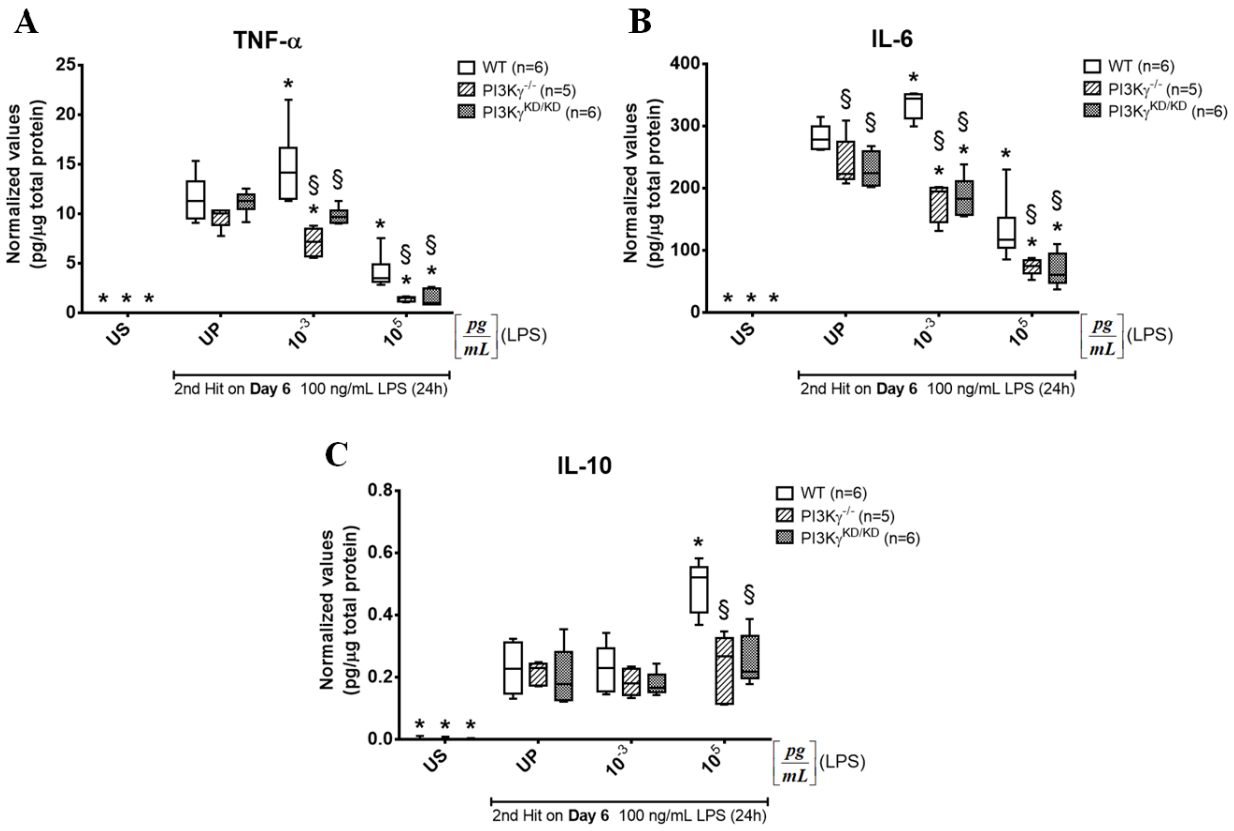

Figure 13: Lipid kinase-dependent function of PI3Kγ controlling the cytokine production during trained sensitization and tolerance induced by sequential challenge with LPS.
Microglial cells were stimulated using the "two hit" approach, priming with 1 fg/mL or 100 ng/mL LPS, and re-stimulated with 100 ng/mL LPS. Low-dose LPS induces increased production of pro-inflammatory cytokines, TNF-α (A) and IL-6 (B), on wild-type microglial cells, whereas knock-out ($PI3K\gamma^{-/-}$) and kinase-dead ($PI3K\gamma^{KD/KD}$) show attenuated levels and significant differences with wild-type cells. High-dose priming with LPS (100 ng/mL) induced decreased TNF-α (A) and IL-6 (B) release. Furthermore primary microglial cells derived from knock-out and kinase-dead mice showed a more reduced production of cytokines under consideration compared with wild-type microglia. The production of IL-10 (C) has mainly been affected after high-dose priming, where wild-type microglia showed increased levels of the anti-inflammatory cytokine, distinct from $PI3K\gamma^{-/-}$ and $PI3K\gamma^{KD/KD}$ that do not show any significant changes. Cytokine production was measured using ELISA immuno-assay (normalized to the total protein concentrations). Data are shown as boxplots and whiskers, *p <0.05, * vs. unprimed condition and §p <0.05, § vs. wild-type strain, 2-way-ANOVA (Holm-Sidak post-test). US, unstimulated; UP, unprimed.

The role of PI3Kγ regulating the cytokine profile during trained sensitization and tolerance response up to date remained unknown. In order to answer this issue we performed experiments using primary microglial cells originated from newborn mice (P0-P3; 1 to 3 days old mice) from the following mice strains: C57Bl/6J (wild type), $PI3K\gamma^{-/-}$ (knock-out) and $PI3K\gamma^{KD/KD}$ (knock-in). Microglial cells isolated from those different strains have been stimulated using the "two hit" approach, performed as described above (see 3.2.2.). Our data show that PI3Kγ controls the

production of pro-inflammatory (TNF-α, IL-6) and anti-inflammatory (IL-10) cytokines during the adaptive responses in primary microglial cells (Fig. 13). Wild-type cells primed with 1 fg/mL and subsequently challenged with 100 ng/mL showed increased production of TNF-α and IL-6 compared to the two other strains (PI3Kγ$^{-/-}$ and PI3Kγ$^{KD/KD}$), where just wild-type microglia were able to develop trained sensitization at 1 fg/mL compared to their unprimed state.

Microglial cells primed with 100 ng/mL LPS released enhanced levels of IL-10 production in wild-type cells compared to PI3Kγ$^{-/-}$ and PI3Kγ$^{KD/KD}$. Furthermore, solely wild-type cells were able to release increased production of the anti-inflammatory cytokine, IL-10, compared with the unprimed condition.

These results confirm that lipid kinase-dependent activity of PI3Kγ comprised the key regulator function for cytokine profiling at trained sensitization and tolerance, representing a crucial role in controlling these adaptive responses in microglial cells.

4.3. Cytokine production is regulated by TLR4/MyD88-dependent pathway of NFκB activation mediated by PI3Kγ

In order to explore possible molecular mechanisms responsible for increased TNF-α and IL-6 production in wild-type cells primed with 1 fg/mL and for the enhanced production of IL-10 by the microglial cells primed with 100 ng/mL LPS compared to unprimed state and PI3Kγ mutants, we first analyzed the protein levels of p110γ (Fig. 14).

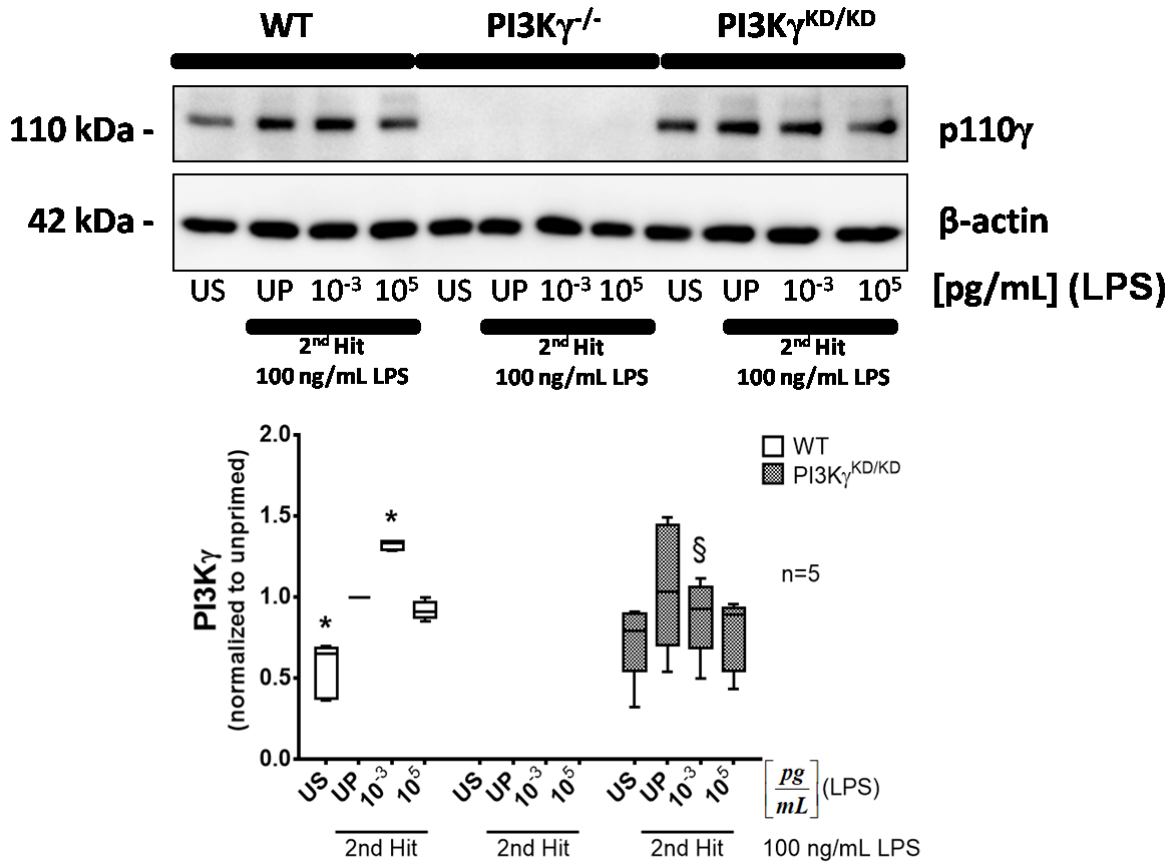

Figure 14: p110γ protein expression in primary microglial cells after priming and subsequent stimulation by LPS.
Primary microglial cells were stimulated as noted above (see 3.2.2.), lysates were analyzed by western blotting technique. Note the increased expression of p110γ in wild-type cells primed with 1 fg/mL LPS, whereas the same condition doesn't show any significant differences in kinase-dead microglial cells compared to unprimed state. Moreover there was increased expression of p110γ in the wild-type cells compared to the kinase-dead cells primed with 1 fg/mL LPS. Data are shown as boxplots and whiskers, *p <0.05, * vs. unprimed condition and §p <0.05, § vs. wild-type strain, 2-way-ANOVA (Holm-Sidak post-test). US, unstimulated; UP, unprimed.

Our data showed that p110γ expression is increased in wild-type microglial cells after priming with 1 fg/mL compared to the unprimed state and thus related with increased levels of pro-inflammatory cytokine (TNF-α, IL-6) production, whereas PI3K$\gamma^{KD/KD}$ microglial cells didn't show any significant expression levels of p110γ compared to unprimed cells. Moreover, wild-type microglial cells primed with 1 fg/mL LPS showed increased levels of p110γ compared to the same condition in the PI3K$\gamma^{KD/KD}$ cells.

Since NFκB activation is one of the main processes for the cytokine production, we further decided to investigate the protein expression of p65 (Rel A), known to regulate the production of

the inflammatory cytokines in different cells. To answer this issue we analyzed the expression of p65 in microglial cells obtained from wild-type, PI3Kγ$^{-/-}$ and PI3Kγ$^{KD/KD}$ mice (Fig. 15).

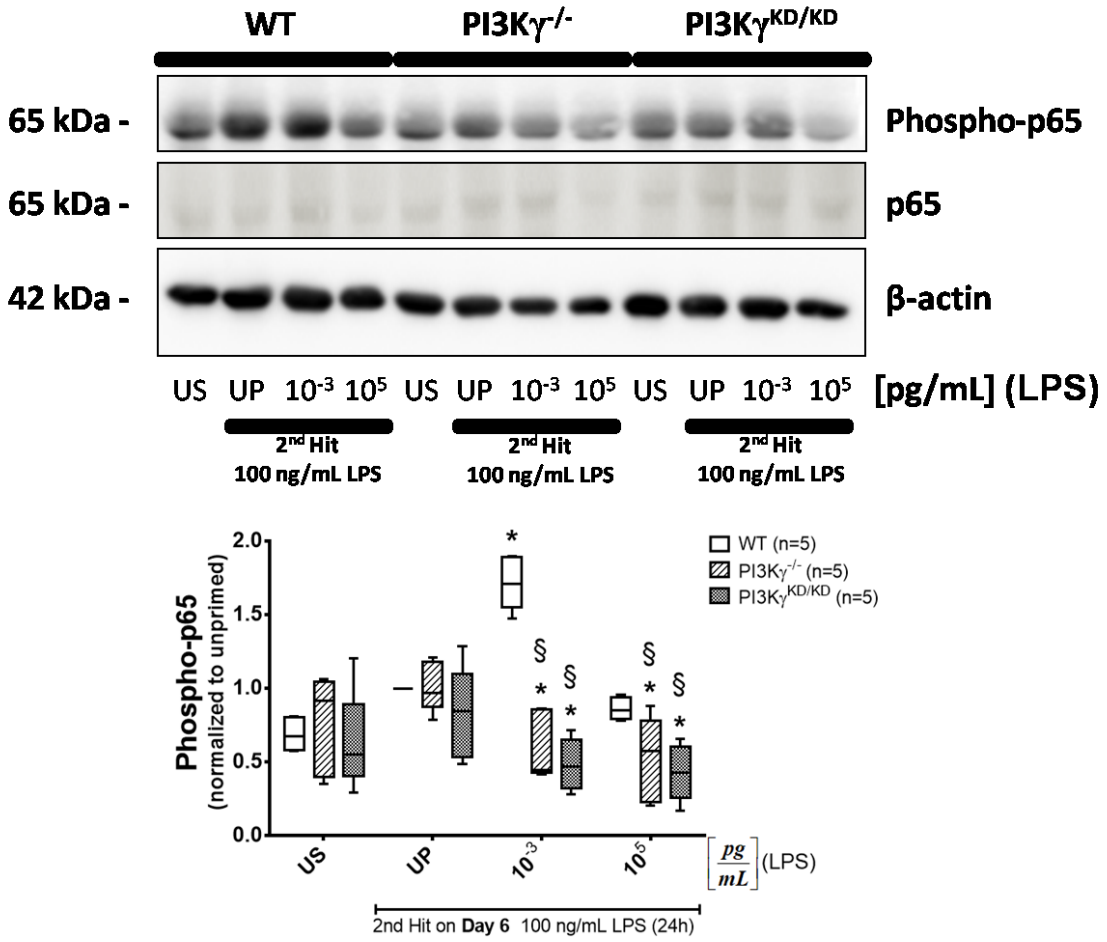

Figure 15: Protein expression of p65 (Rel A) in primary microglial cells after priming and subsequent challenge with LPS.
Microglial cells isolated from different genotypes (wild-type, PI3Kγ$^{-/-}$ and PI3Kγ$^{KD/KD}$) were stimulated using the "two hit" approach as mentioned above, and the lysates have been measured by western blotting method. Note the increased expression of p65 (Rel A) in wild-type cells primed with 1 fg/mL, whereas the same condition showed decreased levels of p65 in the PI3Kγ$^{-/-}$ and PI3Kγ$^{KD/KD}$ cells compared to unprimed condition. Microglial cells primed with 100 ng/mL LPS show decreased protein expression of p65 in PI3Kγ$^{-/-}$ and PI3Kγ$^{KD/KD}$ cells after second LPS challenge compared to the unprimed state. Furthermore wild-type cells show significantly higher expression of p65 compared to other genotypes (PI3Kγ$^{-/-}$ and PI3Kγ$^{KD/KD}$). Data are shown as boxplots and whiskers, *p <0.05, * vs. unprimed condition and §p <0.05, § vs. wild-type strain, 2-way-ANOVA (Holm-Sidak post-test). US, unstimulated; UP, unprimed.

Our results revealed that p65 expression in wild-type cells primed with the low-dose LPS express higher levels of the phosphorylated form of p65, whereas PI3Kγ$^{-/-}$ and PI3Kγ$^{KD/KD}$ microglial cells display reduced expression compared to the unprimed state. PI3Kγ$^{-/-}$ and

PI3K$\gamma^{KD/KD}$ cells primed with high-dose LPS showed decreased levels of the phosphorylated p65 compared to the unprimed state. Moreover wild-type cells displayed increasing protein levels of phosphorylated p65 for both of priming doses, compared with PI3K$\gamma^{-/-}$ and PI3K$\gamma^{KD/KD}$ cells (Fig. 15).

Another member of the NFκB family, Rel B, was reported to suppress the cytokine release of the pro-inflammatory cytokines such as TNF-α and IL-6. In order to investigate the role of Rel B in primary microglial cells after priming and subsequent challenge with LPS, we analyzed the protein expression (Fig. 16).

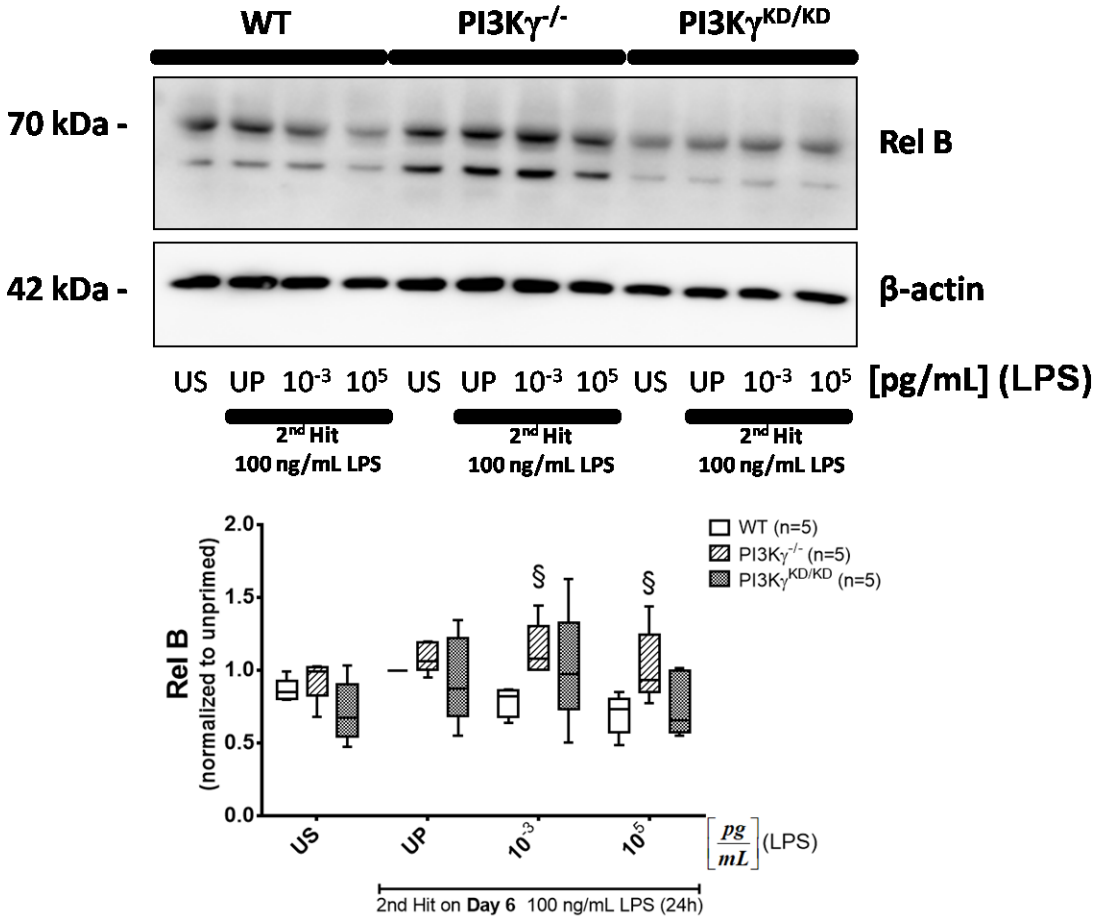

Figure 16: Rel B protein expression in primary microglial cells after priming and subsequent stimulation with LPS.
Wild-type, PI3K$\gamma^{-/-}$ and PI3K$\gamma^{KD/KD}$ microglial cells were stimulated using the "two hit" approach as mentioned above. Lysates have been analyzed by western blotting. Note the increased protein expression of Rel B in the PI3K$\gamma^{-/-}$ cells primed with 1 fg/mL and 100 ng/mL LPS, compared to the wild-type microglia. Data are shown as boxplots and whiskers, *p <0.05, * vs. unprimed condition and §p <0.05, § vs. wild-type strain, 2-way-ANOVA (Holm-Sidak post-test). US, unstimulated; UP, unprimed.

Data revealed that PI3K$\gamma^{-/-}$ microglial cells primed with low and high LPS doses showed increased levels of protein expression for Rel B compared to wild-type genotype. Interestingly, for both priming doses wild-type cells showed a tendency for decreased protein expression levels of Rel B.

These results showed that the cytokine profile is mainly regulated by the interplay between p65 and Rel B activation, and is apparently regulated by PI3Kγ activity in primary microglial cells. Besides it remains to clarify, if the MyD88-dependent or -independent pathway mediates the adaptive response in microglial cells after priming and subsequent stimulation with LPS. In order to investigate this issue RNA samples were collected solely from wild-type cells since these cells were capable to induce both, trained sensitization and tolerance response. Therefore, mRNA levels of the TLR4 receptor and its different adaptor proteins MyD88 (MyD88-dependent) and TRIF (MyD88-independent) have been analyzed using real-time PCR (Fig. 17).

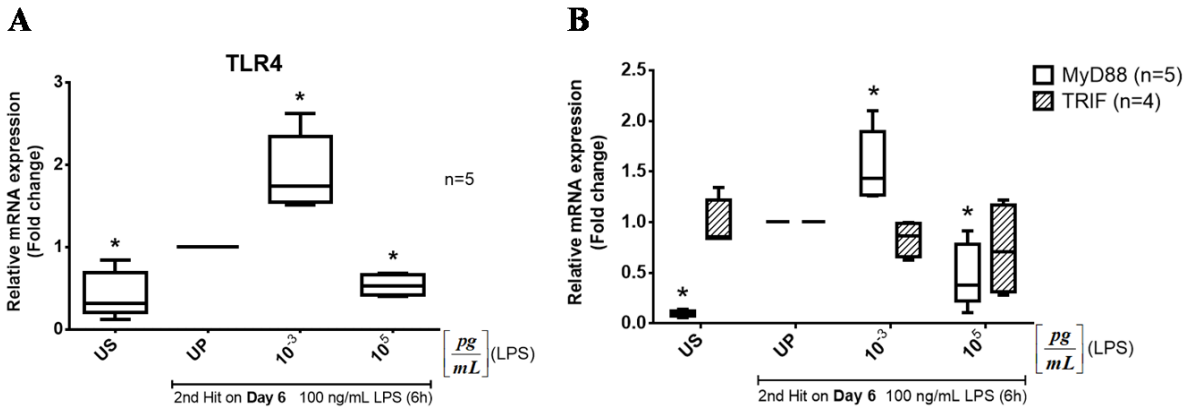

Figure 17: Increased expression of TLR4 at trained sensitization, mediated by the MyD88-dependent pathway, whereas at tolerance a decreased TLR4/MyD88 expression occurred.
TLR4 and MyD88 gene expression was increased in wild-type microglial cells primed with low-dose LPS, whereas priming with high-dose LPS provoked down-regulation of TLR4/MyD88 after subsequent stimulation with LPS. Moreover, TRIF adaptor expression was not significantly affected. Real-time PCR data are shown as boxplots and whiskers, *p <0.05, * vs. unprimed condition, 2-way-ANOVA (Holm-Sidak post-test). US, unstimulated; UP, unprimed; mRNA, messenger RNA.

The above data demonstrated that wild-type microglia primed with 1 fg/mL LPS, show increased mRNA expression of TLR4 as well as significant enhanced MyD88 adaptor expression. In contrast, microglial cells primed with 100 ng/mL LPS showed decreased levels of TLR4/MyD88 expression. The mRNA expression levels of TRIF showed a slightly higher tendency compared to MyD88 at 100 ng/mL, but remained unchanged. Taken together, the above results show that

mainly the TLR4/MyD88-dependent pathway is activated in consequence of priming of microglial cells and subsequent re-challenge with LPS, which is transmitted by p65 and Rel B activation and regulated by the activity of PI3Kγ.

4.4. Trained sensitization and tolerance are characterized by metabolic changes mediated by the lipid kinase-independent activity of PI3Kγ

In the beginning it was reported that "memory-like" adaptive responses of the innate immune system are mainly induced by epigenetic modifications and regulated by metabolic changes. Our previous results have shown that lactate production is markedly increased as a result of trained sensitization and decreased in consequence of tolerance (Fig. 12). In order to investigate role and extent of metabolic changes related with trained sensitization and tolerance we performed measurements of cellular metabolism in microglial cells. This assay allowed us to analyze simultaneously in real-time the glycolytic activity and mitochondrial respiration in primary microglial cells. Furthermore, we investigated the basal – cells at starting assay conditions, as well as stressed - cells under an induced energy demand (specifically, in the presence of stressor compounds such as oligomycin, 2,4-dinitrophenol and antimycin A) phenotypes of OCR and ECAR.

Measurement of oxygen consumption rates (OCR) revealed that microglial cells derived from PI3Kγ$^{-/-}$ mice exhibited an increased oxidative metabolism compared to wild-type cells (Fig. 18). Interestingly, wild-type microglia primed with 1 fg/mL LPS showed increased values of OCR compared to the unprimed state under baseline as well as under stressed condition. This was, different from the other two genotypes (PI3Kγ$^{-/-}$ and PI3Kγ$^{KD/KD}$) under consideration. PI3Kγ$^{KD/KD}$ cells primed with 100 ng/mL LPS showed decreased OCR values under basal condition compared to wild-type cells. Moreover PI3Kγ$^{-/-}$ and PI3Kγ$^{KD/KD}$ cells primed with 100 ng/mL LPS displayed significantly decreased baseline OCR levels compared to the unprimed state. These findings were different from wild-type cells that showed just a tendency of decline (Fig. 18). In summary the data showed that the scaffold function of PI3Kγ appears to be involved in regulating OCR in microglial cells.

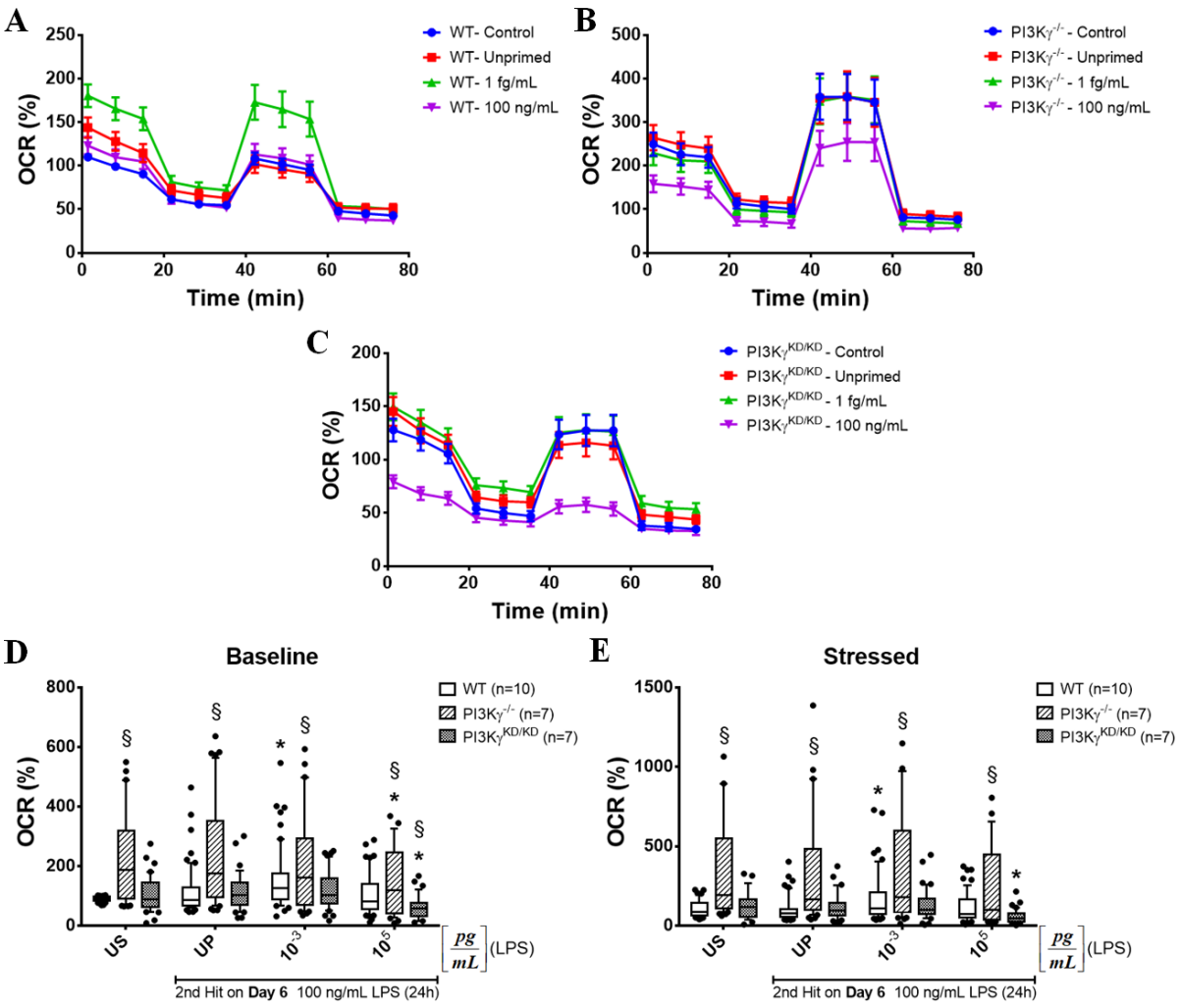

Figure 18: Time-dependent effects on the oxygen consumption rates (OCR) in primary microglial cells after priming and subsequent stimulation with LPS.
Cells have been seeded in 6 replicates per experiment and stimulated as mentioned before using the "two hit" approach, measured by the Seahorse assay. Overall the PI3Kγ$^{-/-}$ (B) genotype of the microglial cells showed enhanced levels of OCR compared to the wild-type (A) and PI3Kγ$^{KD/KD}$ (C) under baseline and stressed phenotype (D, E). Wild-type microglia primed with 1 fg/mL display increased oxygen consumption rates compared to UP state under baseline (D) as well as stressed (E) condition. PI3Kγ$^{-/-}$ and PI3Kγ$^{KD/KD}$ cells show significant decreased OCR values by priming with 100 ng/ml LPS (D, E). Data are normalized to the corresponding cell densities and shown as XY time-dependent graphs (A, B, C) as well quantified and showed as boxplots and whiskers (D, E), *p <0.05, * vs. unprimed condition (2-way-ANOVA) and §p <0.05, § vs. wild-type strain (1-way-ANOVA), Holm-Sidak post-test. US, unstimulated; UP, unprimed; OCR, oxygen consumption rates.

Initially we showed that trained sensitization in microglial cells exhibits increased lactate production suggesting a glycolytic metabolic state. In order to verify that findings the extracellular acidification rates (ECAR) were analyzed. Our results showed that ECAR studied

under stressed conditions exhibited that wild-type microglial cells primed with 1 fg/mL LPS showed significantly enhanced levels of ECAR (Fig. 19). In contrast, PI3Kγ$^{-/-}$ and PI3Kγ$^{KD/KD}$ microglial cells primed with 100 ng/mL LPS display significantly decreased levels of baseline ECAR. Furthermore PI3Kγ$^{-/-}$ cells showed increased ECAR values just under stressed conditions compared to wild-type cells (except under priming with 1 fg/mL).

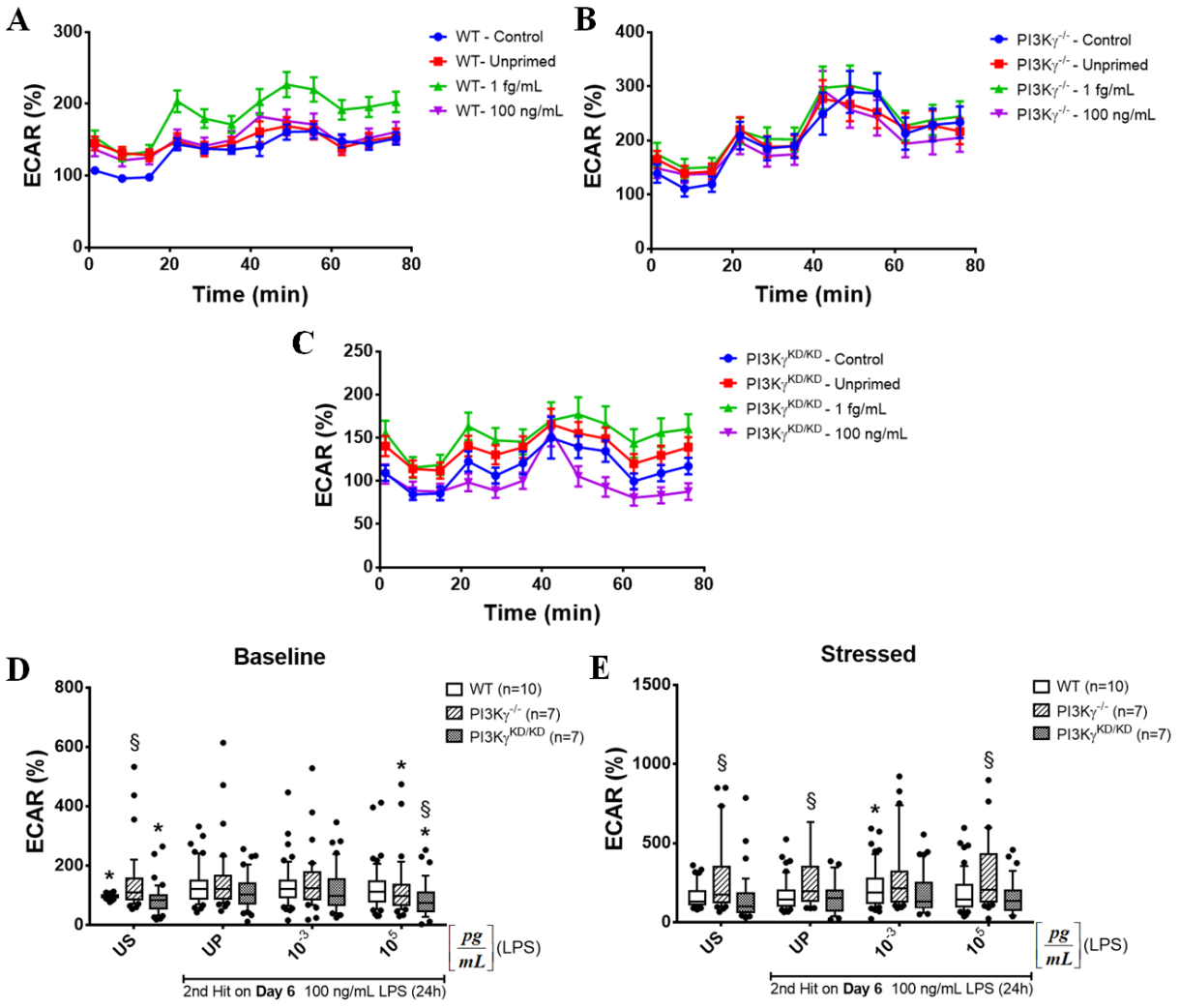

Figure 19: Time-dependent effects on the extracellular acidification rates (ECAR) in primary microglial cells after priming and subsequent stimulation with LPS.
Cells have been seeded in 6 replicates per experiment and stimulated as mentioned before using the "two hit" approach, measured by the Seahorse assay. Overall the PI3Kγ$^{-/-}$ (B) microglial cells show enhanced levels of ECAR compared to the wild-type (A) and PI3Kγ$^{KD/KD}$ (C) microglial cells under the stressed conditions (E), but not under baseline conditions (D). Wild-type microglia primed with 1 fg/mL display increased extracellular acidification rates compared to UP state under stressed (E), but not in the baseline (D) condition. PI3Kγ$^{-/-}$ and PI3Kγ$^{KD/KD}$ cells showed significantly decreased ECAR values by priming with 100 ng/ml LPS (D). ECAR values were normalized to the corresponding cell densities and the data

are shown as XY time-dependent graphs (A, B, C) as well quantified and showed as boxplots and whiskers (D, E), *p <0.05, * vs. unprimed condition (2-way-ANOVA) and §p <0.05, § vs. wild-type strain (1-way-ANOVA), Holm-Sidak post-test. US, unstimulated; UP, unprimed; ECAR, extracellular acidification rate.

The above data revealed that the lipid kinase-independent activity of PI3Kγ plays a certain role in controlling glycolysis in primary microglial cells (Fig. 19).

Based on the OCR and ECAR values, related metabolic parameters, such as ATP production, non-mitochondrial respiration, proton leakage and spare capacity were calculated in order to characterize further the impact of PI3Kγ on the energy metabolism of microglial cells under different conditions. Our data show that PI3Kγ$^{-/-}$ cells displayed increased levels of all the parameters compared with wild-type microglia (Fig. 20).

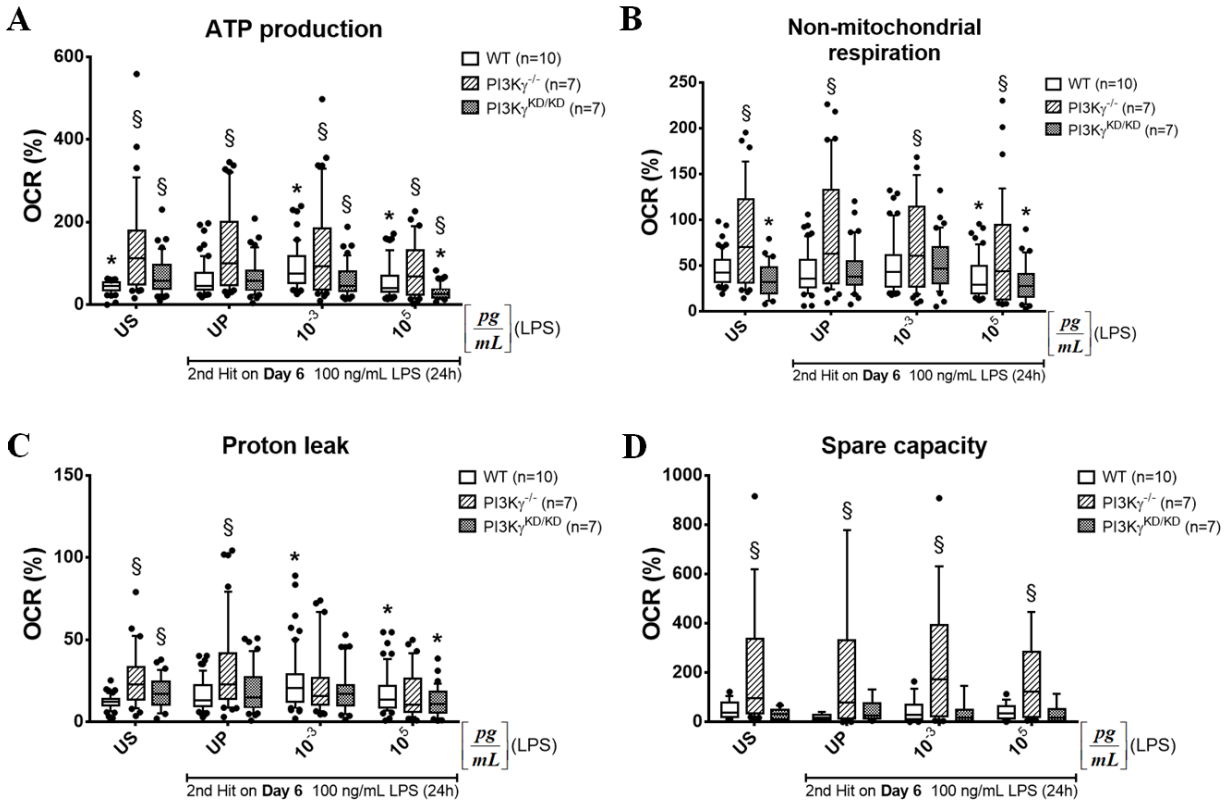

Figure 20: Respiratory metabolic parameters are controlled by the lipid kinase-independent activity of PI3Kγ.
ATP production (A), non-mitochondrial respiration (B), proton leak (C) and spare capacity (D) are mediated by the scaffold function of PI3Kγ, characterized with increased amounts in PI3Kγ$^{-/-}$ microglial cells compared to wild-type cells. Furthermore wild-type cells primed with 1 fg/mL express increased amounts of ATP production (A) as well as proton leak (C). PI3Kγ$^{KD/KD}$ as well wild-type cells primed with 100 ng/mL LPS showed decreased expression of all the parameters (A, B, C), except the spare

capacity (D). Data are shown as boxplots and whiskers, *p <0.05, * vs. unprimed condition (2-way-ANOVA) and §p <0.05, § vs. wild-type strain (1-way-ANOVA), Holm-Sidak post-test. US, unstimulated; UP, unprimed; OCR, oxygen consumption rates.

These data showed that during trained sensitization, wild-type microglial cells exhibited an increased ATP production (by 1,45 times) and proton leak (by 1,53 times), whereas the cells with the other genotypes under consideration do not show any differences under the same condition compared to the unprimed state. Under tolerance response wild-type as well PI3Kγ$^{KD/KD}$ microglial cells showed decreased amounts of ATP production, non-mitochondrial respiration as well as the proton leakage (Fig. 20). These data showed also that mitochondrial activity is involved in the induction of different adaptive responses controlled by PI3Kγ in microglial cells. Taken together these data revealed the crucial importance of metabolic changes inducing both of adaptive responses (trained sensitization and tolerance), tightly regulated by the lipid kinase-independent activity of PI3Kγ.

4.5. PI3Kγ regulates phagocytic activity during tolerance response in microglial cells *in vitro*

Phagocytosis represents one of the main essential functions of the microglial cells in the CNS under healthy (e.g. synapse pruning) and pathological conditions (e.g. debris removal) in order to maintain or re-establish basic neurologic functions. In order to verify if trained sensitization or tolerance as adaptive immune responses induced after priming and subsequent stimulation with LPS could influence such a complex microglial function like phagocytosis, we performed the *in vitro* phagocytosis assay.

Microglial cells derived from mice with different genotypes (wild-type, PI3Kγ$^{-/-}$ and PI3Kγ$^{KD/KD}$) were used and prepared as mentioned (see 3.2.10.), and the phagocytic activity was assessed by adding Zymosan- FITC labeled particles. As shown in Fig. 21 trained sensitization provoked in wild-type microglial cell a marked reduction of phagocytic activity compared to the unprimed condition, whereas the tolerant state displayed an increased phagocytosis. PI3Kγ$^{-/-}$ cells displayed a significantly decreased phagocytosis indicating a lipid kinase-independent activity of PI3Kγ, because PI3Kγ$^{KD/KD}$ cells showed mainly a similar behavior as seen in wild-type cells.

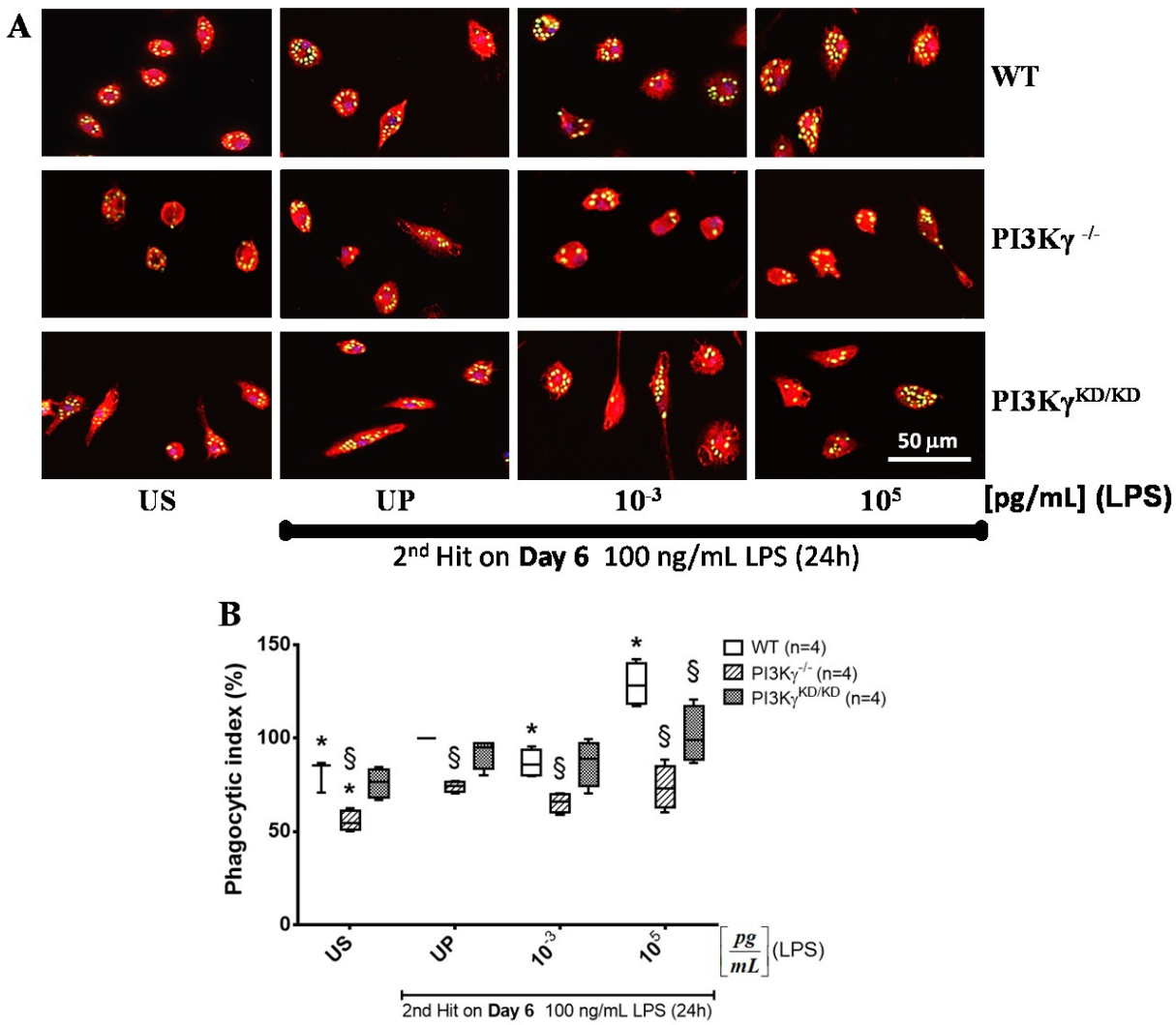

Figure 21: PI3Kγ controls the phagocytic activity during trained sensitization and tolerance in primary microglial cells.

Primary microglial cells were stimulated as mentioned above using the "two hit" approach. Phagocytosis of the FITC labeled Zymosan particles (green dots) has been quantified as shown (see 3.2.10.). Iba1 (a marker of microglial cells) labels primary microglial cells (red color) and DAPI (blue) the nucleus of the cells. (A) Representative images of phagocytosis of FITC Zymosan particles in wild-type, PI3Kγ$^{-/-}$ and PI3Kγ$^{KD/KD}$ primary microglial cells *in vitro*. (B) Quantification of phagocytosis represented as phagocytic index (the uptake rate of FITC-Zymosan particles per cell). Data are shown as boxplots and whiskers, *p <0.05, * vs. unprimed condition and §p <0.05, § vs. wild-type strain, 2-way-ANOVA (Holm-Sidak post-test). US, unstimulated; UP, unprimed.

4.6.PI3Kγ mediates phagocytosis and metabolic changes by lipid kinase-independent control of cAMP

Taking under consideration that the scaffold function of PI3Kγ and its impact on microglial cAMP signaling appears to be a key mediator of metabolic changes and phagocytic activity in microglial cells during trained sensitization and tolerance, we intended to assess intracellular cAMP content in microglial cells under these conditions. As shown in Fig. 22, increased levels of cAMP are displayed in PI3Kγ$^{-/-}$ microglial cells, compared to the wild-type cells. Furthermore, a significant cAMP reduction occurred in wild-type cells after high dose priming and re-stimulation.

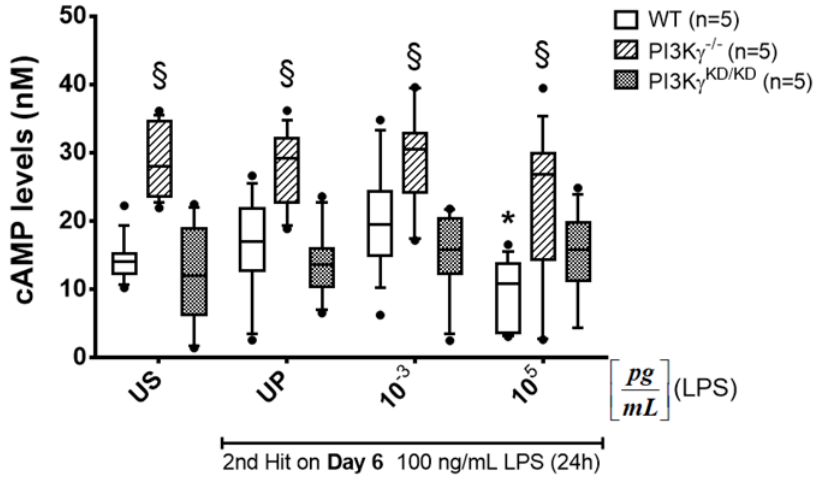

Figure 22: The effect of PI3Kγ on the cAMP levels during adaptive responses in primary microglial cells.
Microglial cells (wild-type, PI3Kγ$^{-/-}$ and PI3Kγ$^{KD/KD}$) have been stimulated using the "two hit" approach and the cAMP levels have been measured using the cAMP GloAssayKit. Data are shown as boxplots and whiskers, *p <0.05, * vs. unprimed condition and §p <0.05, § vs. wild-type strain, 2-way-ANOVA (Holm-Sidak post-test). US, unstimulated; UP, unprimed.

4.7. PI3Kγ mediates phagocytosis during tolerance development *in vivo*

In order to verify the biological role of a sustained impact of PI3Kγ on phagocytosis owing to induced tolerance, an animal experiment has been performed.

Data indicate a quite similar behavior of microglial cells in the brain as documented under cell culture conditions. Most strikingly, in brains of wild-type mice with induced microglial tolerance

behavior was found an enhanced phagocytic activity (Fig. 23). Furthermore, microglial cells in brains of PI3K$\gamma^{-/-}$ mice displayed a reduced phagocytic activity as seen in previous studies, as for our *in vitro* phagocytosis, where high doses of LPS induced increased phagocytic activity of microglial cells in the wild-type mice. Furthermore the lipid kinase-independent function of PI3Kγ mediates the phagocytic activity in the microglial cells in brain issue during both of adaptive responses, trained sensitization and tolerance.

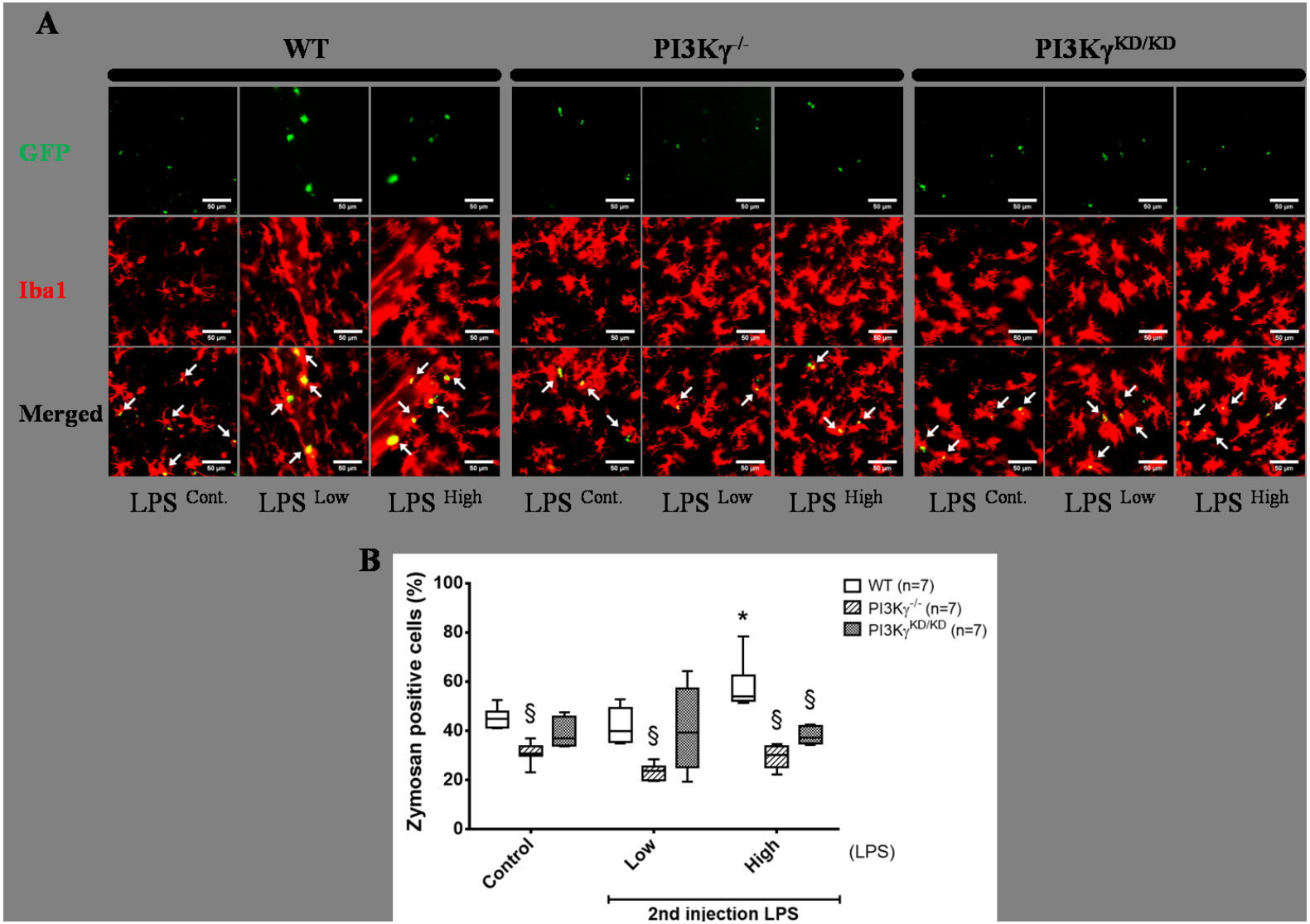

Figure 23: Scaffold function of PI3Kγ mediates phagocytic activity in microglial cells *in vivo*.

3 months old mice (WT, PI3K$\gamma^{-/-}$ and PI3K$\gamma^{KD/KD}$) were used for the experiment. Mice were initially injected with either low-dose LPS (0.025 mg/kg, i.p.) or high dose LPS (10 mg/kg, i.p.) for priming and 3 days later with a subsequent application of 10 mg/kg, i.p. LPS. Phagocytosis of the FITC labeled Zymosan particles has been quantified as mentioned (see. 3.2.11.). Markers such as Iba1 (microglial cells), and GFP (FITC labeled Zymosan particles) were used. (A) Representative images of phagocytosis of FITC Zymosan particles in wild-type, knock-out and kinase-dead primary microglial cells *in vivo*. (B) Quantification results (percentage) of the of Iba-1 positive cells per voxel mm^3 containing Zymosan particles. Data are shown as boxplots and whiskers, *p <0.05, * vs. unprimed condition and §p <0.05, § vs. wild-type strain, 2-way-ANOVA (Holm-Sidak post-test).

Our findings indicate PI3Kγ-dependent suppression of cAMP signaling as a critical regulatory element of microglial phagocytosis *in vitro* as well as *in vivo*.

4.8. Cytokine profile in blood plasma and brain tissue are regulated by kinase activities of PI3Kγ

Initially we showed under *in vitro* conditions that cells primed with low-dose LPS induce increased production of pro-inflammatory cytokines - trained sensitization, different from high-dose primed microglia that induced a tolerance response characterized with decreased production of TNF-α and IL-6. Furthermore we showed that lipid kinase-dependent activity of PI3Kγ mediates the cytokine production in microglial cells.

In order to investigate the role of PI3Kγ in regulating cytokine production under conditions of trained sensitization as well as tolerance induced under *in vivo* conditions, the cytokine profile was estimated in blood and brain tissue obtained from mice after priming with low as well as high doses of LPS in 3 month old mice (WT, PI3Kγ-/- and PI3KγKD/KD). As shown in Fig. 24, TNF-α and IL-6 produced in the blood plasma after subsequent injection displayed increased levels in wild-type compared to PI3K$\gamma^{-/-}$ and PI3K$\gamma^{KD/KD}$, representing as shown above *in vitro* the importance of lipid kinase-dependent function of PI3Kγ mediating the cytokine profile *in vivo*. In addition, brain cytokines (TNF-α, IL-6) were affected also by the kinase activity of PI3Kγ, especially in the low-dose injected mice strains.

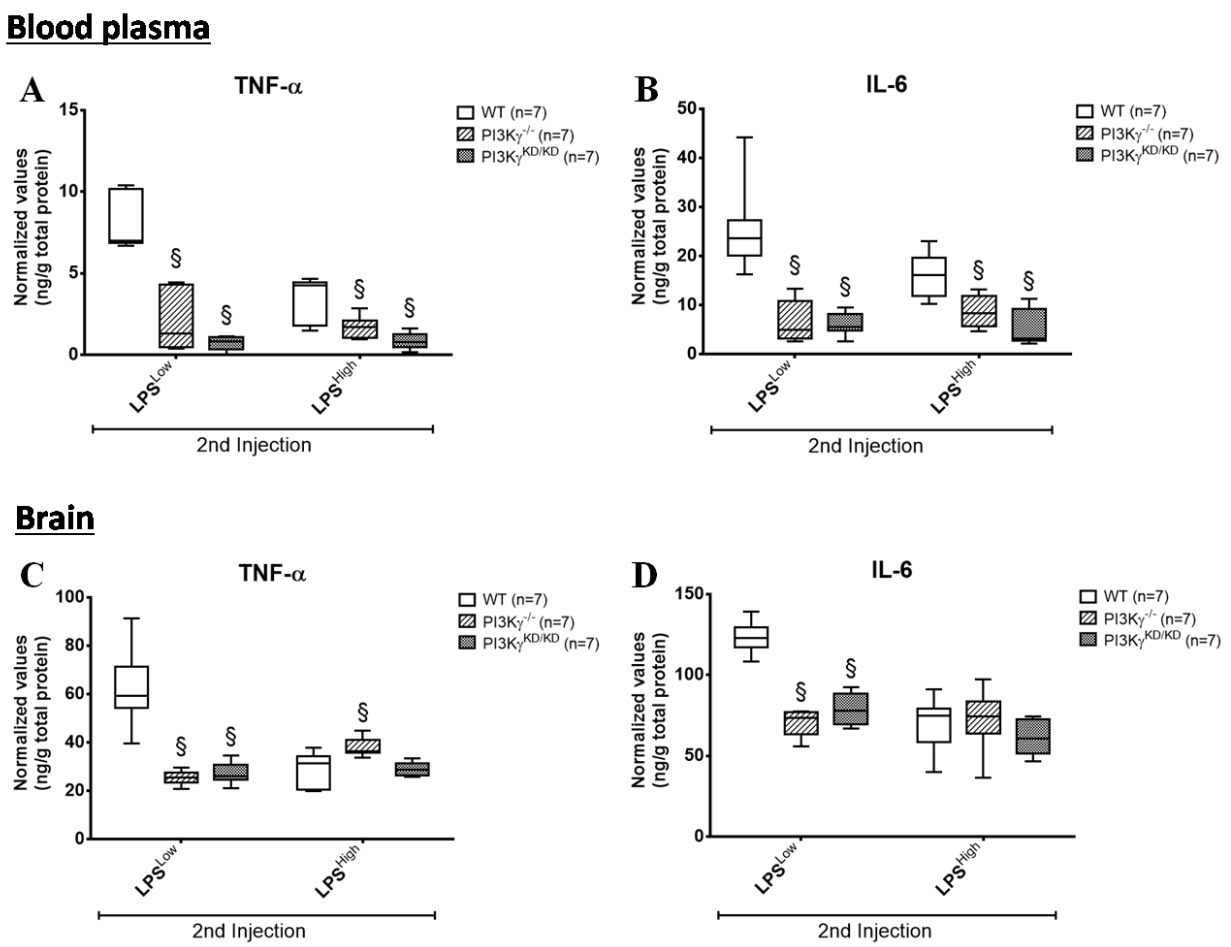

Figure 24: Lipid kinase-dependent role of PI3Kγ regulates the cytokine production in blood plasma and brain tissue.
3 months old mice (WT, PI3Kγ$^{-/-}$ and PI3Kγ$^{KD/KD}$) were used for the experiment. They were initially injected with either low dose LPS (0.025 mg/kg, i.p.) or high dose LPS (10 mg/kg, i.p.) for priming and 3 days later with a subsequent application of 10 mg/kg, i.p. LPS. Cytokine production (normalized to total protein concentration) in the blood plasma (A, B) and brain (C, D) were assayed using ELISA immuno-assay. Data are shown as boxplots and whiskers, §p <0.05, § vs. wild-type strain, 1-way-ANOVA (Holm-Sidak post-test).

Taken together, our results showed that lipid kinase-dependent function of PI3Kγ is a key mediator of the cytokine profile in the blood plasma and brain tissue, after subsequent administration of LPS.

V. Discussion

5.1. Methodical considerations

There is possibly some concern when recognized that rather low doses of stressors from microbial origin are potent to induce relevant priming effects in naïve microglial cells. Nevertheless, own data clearly show that priming with 1 fg/mL LPS was able to induce a significant increase of TNF-α and IL-6 release after repeated contact with an increased LPS dose 5 days after the first PAMP contact (see Fig. 5 and 8). In order to verify these findings regarding dose dependency, we performed experiments targeting estimation of lower limit of LPS concentration which is potent to induce trained sensitization. As clearly shown in Fig. 25 a LPS concentration just below the dosage used to induce priming for trained sensitization (e.g., 0.3 fg/mL) as well as a stepwise higher dose (e.g., 3 fg/mL) failed to induce an enduring state of trained sensitization in cultured microglial cells. Therefore, the dose of 1 fg/mL remained the optimal priming dose to induce significant production of different inflammatory mediators.

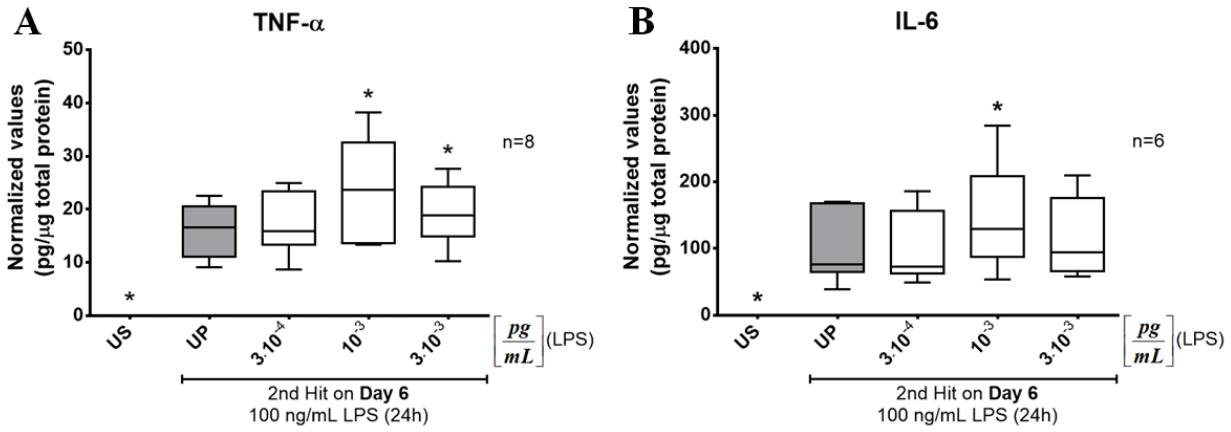

Figure 25: LPS dose investigations for the induction of trained sensitization in microglial cells.
Microglial cells have been stimulated using "two hit" approach and analyzed with ELISA immuno-assay (normalized to total protein concentration) for: TNF-α (A) and IL-6 (B) production. Data are shown as boxplots and whiskers, *p <0.05, * vs. unprimed condition (grey column), 2-way-ANOVA (Holm-Sidak post-test). US, unstimulated; UP, unprimed.

Besides, there is probably an age-dependent modification of priming sensitivity as reported previously (Godbout et al. 2005). Own unpublished data show that – in contrast to microglial cells harvested from newborn mice, used in this study - cultured microglial cells obtained from

adult mouse brain did not show trained sensitization, whereas tolerance was partly more pronounced (Lajqi et al. in prep.). These data suggest that microglial cells, probably with missing previous PAMP contact (e.g. really naïve cells) exhibit an extremely high sensitivity for TLR-dependent activation (Nikodemova et al. 2016), whereas resident microglial cells with presumably a certain history of PAMP (as well as DAMP) contacts develop possibly a (long-lasting) tolerance.

Interestingly, the dose of 1 fg/mL LPS represents about 122.868 molecules of LPS priming microglial cells, thus inducing trained sensitization after subsequent challenge. Several studies have shown that TLR4 can sense LPS concentrations in a range of picomolar down to femtomolar (Morin et al.; Dinarello 1992; Wilson et al. 2002; Paramo et al. 2016; Civciristov et al. 2018). This sensitivity of TLR4 is mainly adapted by the co-activity of anchor proteins such as cluster of differentiation 14 (CD14) and MD-2 (Dziarski and Gupta 2000; Gioannini et al. 2004). Furthermore, microglial cells survey in a microenvironment - central nervous system, which is markedly protected from the outside environment including circulating blood, where occurrence of certain values of PAMPs like LPS has to be assumed. Therefore, it is not surprising that naïve (newborn) microglial cells display an increasing affinity to sense low PAMP doses and being more susceptible than blood-born immune cells (White et al. 1998).

Our research study identifies the pathogen-dose as the critical mediator for the induction of both of adaptive responses, trained sensitization and tolerance in microglial cells. Furthermore this study gives more insights about the sensitivity of microglial cells memorizing very low levels of different PAMPs.

5.2. Discussion of research findings

5.2.1. Dose-dependent induction of trained sensitization and tolerance in microglial cells

Current investigations demonstrated that the innate immune cells (monocytes, macrophages and brain resident macrophages- microglia) can mount a memory-like behavior, principally similar to the immunological memory of the acquired arm after subsequent infection that has been termed as trained sensitization (Ifrim et al. 2014; Arts et al. 2016a). In order to prevent any misunderstanding with the actual used term of trained immunity which was conceptualized as long-term effect, we used the term trained sensitization which mainly represents a state of microglial cells displaying increased production of pro-inflammatory cytokines (TNF-α, IL-6) by sensing different pathogen ligands at a specific time-point (Bauer et al. 2018). In contrast, tolerance (desensitization) response was characterized with reduced production of pro-inflammatory cytokines followed by increased anti-inflammatory response after repeated challenge by high loads of LPS (Sly et al. 2004). Only few studies have investigated long-term microglial functions as a result of a single immunostimulatory challenge, but none of them in the context of microglia displaying trained sensitization or tolerance in a dose-dependent manner after subsequent stimulation by different PAMPs (LPS, β-glucan). Our data showed that single stimulation by LPS, as well β-glucan, of the microglial cells induces a dose-dependent induction of pro-inflammatory cytokines (TNF-α, IL-6) (Fig. 4, 6A). Corresponding studies showed that monocytes sensitize priming with low-dose LPS, after repeated challenge inducing increased release of pro-inflammatory mediators such as cytokines (TNF-α, IL-6) and reactive oxygen species (ROS) (Chen et al. 2015; Yuan et al. 2016). Current data in microglial cells showed dose-dependent relation, where priming with low doses (especially at 1 fg/mL) LPS, after re-challenge induced trained sensitization characterized by increased production of the pro-inflammatory cytokine TNF-α, ROS and iNOS (Fig. 5). In contrast, priming with higher-doses (especially at 100 ng/mL) of LPS, after re-challenge induced prolonged reduction of the previous mentioned inflammatory mediators- tolerance response. Different studies postulated that priming of innate immune cells is shaped only by specific pathogen molecules such as β-glucan and BCG (Buffen et al. 2014; Ifrim et al. 2014; Saeed et al. 2014). Similarly, β-glucan primed microglial cells showed dose-dependent adaptive responses, where low-doses (especially 100 fg/mL)

triggered enhanced levels of TNF-α, whereas higher doses (especially 1 μg/mL) led to reduced amounts of TNF-α (Fig. 6, 7). The resulted biphasic effects by which low doses trigger stimulatory responses and high-dose results with suppressive responses has been termed hormesis (Calabrese and Baldwin 2001). Indeed, microglial cells adapt to different pathogen ligands (LPS, β-glucan) after re-challenge inducing biphasic hormetic responses where low doses led to increased production of pro-inflammatory mediators, whereas higher doses displayed decreased levels of the inflammatory response as shown (Fig. 5, 6, 7). Furthermore, microglial cells primed by different PAMPs (LPS, β-glucan) showed attenuated production levels of IL-6, after repeated challenge by LPS, where low-dose priming induced trained sensitization response, whereas high-dose priming triggered the tolerance development (Fig. 8, 9).

Occurrence of the trained sensitization as a resistance mechanism was reported to be dependent on the expression of HIF-1α in monocytes (Cheng et al. 2014) followed by increased lactate production promoting a metabolic state toward glycolysis. Several investigations have shown that trained sensitization as well as tolerance are mediated by epigenetic changes followed by metabolic modifications (Saeed et al. 2014; Arts et al. 2016b; Liu et al. 2016). Microglial cells primed with low-dose LPS expressed similar phenotype as monocytes inducing increased expression of HIF-1α (Fig. 10B) characterized by increased lactate amounts and PFKFB3 expression (Fig. 12) promoting the shift toward glycolysis and being responsible for the induction of trained sensitization. Meanwhile, a study showed that microglial cells re-challenged by high loads of LPS display a tolerance response as a consequence characterized with decreased levels of inflammatory mediators (Schaafsma et al. 2015). Moreover, this state of tolerance is further characterized by increased levels of anti-inflammatory mediators, especially IL-10 and TGF-β (Nomura et al. 2000). Interestingly, results showed that priming with 100 ng/mL LPS after repeated challenge led to increased production of IL-10, as well expression of different anti-inflammatory mediators (IL-10, TGF-β, IL-4 and Arg-1) (Fig. 11) followed by decreased production of pro-inflammatory mediators (TNF-α, IL-6, ROS, iNOS) as shown above and decreased lactate amounts (Fig. 12A). A recent complementary study done on microglial cells *in vivo* reported similar immune adaptive responses, where microglia primed with low-dose LPS displayed trained sensitization and higher dose repeated stimulations with LPS developed a tolerant state, thus affecting the formation of β-amyloid plaques (Wendeln et al. 2018).

Furthermore our data indicated that trained sensitization is mainly characterized by increased expression and production of the pro-inflammatory mediators with a shift toward glycolysis as metabolic state characterized by increased lactate production, and thus representing phenotype characteristics toward “M1”-like polarization (Kobayashi et al. 2013; Orihuela et al. 2016). Moreover, tolerance displayed reduced production of inflammatory markers and lactate in addition to increased expression levels of the anti-inflammatory mediators, exhibiting characteristics of “M2”-like phenotype of microglia (Landau et al. 2010; Avdic et al. 2013; Colonna and Butovsky 2017). Comparable findings have been reported where endotoxin tolerance represents a distinctive alternative activation of macrophages (M2 phenotype) (Pena et al. 2011).

Taken together, these data underline the key importance of the sensing dose of stressors (here PAMPs) such as LPS and β-glucan for the development of innate immune-mediated adaptive responses after subsequent challenge by the same or different conserved structures of different pathogens, resulting with trained sensitization by low-dose priming and tolerance by high-dose priming of microglial cells.

5.2.2. Lipid kinase-dependent and -independent activities of PI3Kγ mediate trained sensitization and tolerance in microglial cells

Up to date, different investigations have reported a tight interplay of PI3Ks family regulating the induction of trained sensitivity by activating Akt/mTOR/NFκB pathway as well as tolerance by suppressing this pathway (Cheng et al. 2014; Poplutz et al. 2017; Xu et al. 2018). Furthermore, phosphoinositide 3-kinase γ (PI3Kγ) was known to mediate stress-induced immune responses (Jin et al. 2011). Our group reported that PI3Kγ is a key regulator of different cellular processes in primary microglial cells affecting the inflammatory response in addition to biological developments in the brain (Schmidt et al. 2013; Frister et al. 2014; Schneble et al. 2017). Latest reports showed a lipid kinase-dependent function of PI3Kγ regulating the cytokine production profile on macrophages (Luo et al. 2014; Kaneda et al. 2016). Our data showed that the induction of adaptive responses in microglial cells mainly characterized by different attitudes of the cytokine profile, in one side increased production of the pro-inflammatory cytokines (TNF-α, IL-

6) by low-dose priming with LPS – trained sensitization, and in the other side with reduced inflammatory response, but enhanced levels of the anti-inflammatory cytokines such as IL-10 by priming with high-dose LPS – tolerance, were mediated by the lipid kinase-dependent activity of PI3Kγ (Fig. 13). Furthermore, extensive protein expression of PI3Kγ (Fig.14) triggered increasing production of TNF-α and IL-6 during trained sensitization in wild-type microglia, different from PI3K$\gamma^{-/-}$ and PI3K$\gamma^{KD/KD}$ cells that showed reduced cytokine production levels.

Activation of the NFκB family, especially Rel A (p65) promotes the release of different inflammatory mediators (Kwon et al. 2016). Our data showed that the molecular mechanism behind the induction of trained sensitization in primary microglial cells relates on the p65 activation, triggering the increased production of TNF-α and IL-6 (Fig. 15). The expression of p65 in wild-type cells during tolerance showed a slightly decrease compared to the unprimed state, leading to increased production of IL-10. Wild-type microglial cells compared to PI3K$\gamma^{-/-}$ and PI3K$\gamma^{KD/KD}$ expressed higher protein levels of p65 during trained sensitization and tolerance, confirming our previous findings about the cytokine production mediated by the lipid kinase-dependent activity of PI3Kγ. Comparable findings were reported in macrophages showing a tight regulation of the p65 activity in the induction of pro- and anti-inflammatory cytokines, mediated by the kinase function of PI3Kγ (Kaneda et al. 2016).

In addition Rel B, another member of the NFκB family, known to suppress the release of cytokines, partially by the inhibition of p65 activity (Gasparini et al. 2013), showed increased protein expression in PI3K$\gamma^{-/-}$ microglial cells compared to wild-type cells, resulting with decreased production of TNF-α, IL-6 and IL-10 (Fig. 16). Notably, PI3K$\gamma^{KD/KD}$ cells show a slightly tendency toward higher protein expression levels of Rel B, resulting with decreased amounts of cytokine production. Interestingly, p65 and Rel B relay on a kind "yin/yang" equilibrium where p65 induced cytokine production is partially suppressed by the activity of Rel B, or vice-versa, influencing the cytokine profile in different cells (Jacque et al. 2005).

TLR4-induced activation by LPS is the key cascade triggering the NFκB translocation into the nucleus and thus leading to the production of pro-inflammatory mediators (Hoesel and Schmid 2013; Liu et al. 2017). Our results showed that only wild-type microglial cells were capable to display both, trained sensitization and tolerance, different from PI3K$\gamma^{-/-}$ and PI3K$\gamma^{KD/KD}$ microglial cells that were able to mediate only tolerance response. Furthermore, our results

showed that both of adaptive responses in microglial cells were induced by TLR4/MyD88-dependent pathway characterized by upregulated expression during trained sensitization and downregulated expression by tolerance (Fig. 17). Similarly, a report showed that downregulation of the surface receptor TLR4 on macrophages followed by the reduced expression of the adaptor gene MyD88 triggers the state of endotoxin tolerance (Nomura et al. 2000).

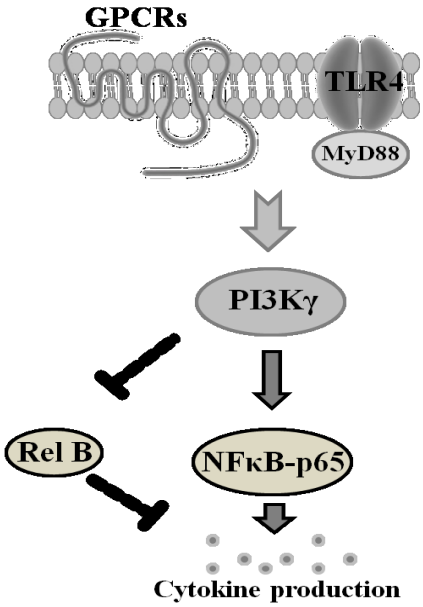

Figure 26: Schematic model depicting the PI3Kγ signaling to promote cytokine production by the activation of NFκB.
Reports have shown that PI3Kγ activation by TLR4 triggers downstream processes promoting NFκB activation, especially p65, thus affecting the cytokine profile in the innate immune cells (Luo et al. 2014; Kaneda et al. 2016). In contrast, Rel B activation suppresses the pro-inflammatory cytokine release (Gasparini et al. 2013).

Our current findings showed for the first time that TLR4/MyD88-dependent activation of NFκB, especially p65, mediated by the lipid kinase-dependent function of PI3Kγ is a critical determinant for the induction of both, trained sensitization and tolerance response in microglial cells.

Up to date, increasing studies indicated epigenetic modifications followed by metabolic changes as main novelties important for the induction of trained sensitization and tolerance in myeloid cells, especially monocytes and macrophages (Buffen et al. 2014; Saeed et al. 2014; Arts et al. 2016a). In addition, as shown above induction of trained sensitization in wild-type microglial cells was characterized with increased lactate production inducing a shift toward glycolysis as

main metabolic process. A recent study revealed that increased oxygen consumption rates (OCR) and extracellular acidification rates (ECAR) represented main metabolic changes characterizing trained sensitization compared to their unprimed state, and thus identifying both metabolic processes, glycolysis and oxidative phosphorylation as main processes affecting the induction of adaptive processes in monocytes (Domínguez-Andrés et al. 2018). Real-time metabolic investigations of microglial cells from different genotypes (wild-type, PI3K$\gamma^{-/-}$ and PI3K$\gamma^{KD/KD}$) showed that wild-type cells capable to induce trained sensitization by low-dose displayed higher baseline as well stressed OCR (Fig. 18), characterized by increasing ATP production and proton leak (Fig. 20 A, C) representing the oxidative phosphorylation as one of the main metabolic changes for the induction of adaptive responses. Furthermore, wild-type microglia primed with low-dose LPS expressed enhanced levels of stressed ECAR (Fig. 19), characterized by increased levels of lactate, shifting the metabolic process toward glycolysis. Moreover, tolerant microglia from PI3K$\gamma^{-/-}$ and PI3K$\gamma^{KD/KD}$ showed significantly decreased levels of baseline OCR and ECAR compared to their unprimed control (Fig. 18, 19), characterized also with reduced levels of cytokine production (TNF-α, IL-6 and IL-10) different from wild-type cells that displayed higher amounts (Fig. 13). Interestingly, PI3K$\gamma^{-/-}$ microglial cells showed increased amounts of baseline and stressed OCR, as well as stressed ECAR values compared to the wild-type cells, indicating a scaffold function of PI3Kγ mediating metabolic activities such as glycolysis and oxidative phosphorylation. Therefore PI3K$\gamma^{-/-}$ cells displayed increased amounts of cAMP production compared to wild-type cells, confirming the lipid kinase-independent activity of PI3Kγ, mediated here by decreased activity of PDE (Fig. 22). Few studies have reported also the importance of the lipid kinase-independent activity of PI3Kγ mediating glycolysis and further hypothesized that could be crucial for the mediation of oxidative phosphorylation (Becattini et al. 2011; Ha et al. 2013). Different metabolic parameters such as ATP production, non-mitochondrial respiration, proton leak and spare capacity were mediated by the scaffold activity of PI3Kγ, defined by increased levels in the PI3K$\gamma^{-/-}$ microglial cells. Furthermore, wild-type and PI3K$\gamma^{KD/KD}$ primed by 100 ng/mL LPS, inducing tolerance showed decreased amounts of ATP production, non-mitochondrial respiration, proton leak and spare capacity, compared to the unprimed control (Fig. 20), displaying increased microglial phagocytic activity, especially in wild-type microglia *in vitro* as well *in vivo* (Fig. 21, 23). Our data indicated that metabolic changes indeed are involved in the mediation of trained sensitization as well tolerance, regulated by the lipid kinase-

independent activity of PI3Kγ in microglial cells. Furthermore our results revealed that mitochondria as main organelles important for the energy support in microglial cells play a key role in the induction of trained sensitization and tolerance, mediated by the scaffold function of PI3Kγ.

Up to date, different reports showed that activation of PI3K signaling pathway is critical for the regulation of epigenetic modifications, especially involved in the methylation of histones and thus triggering different cellular responses (Zuo et al. 2011; Toska et al. 2017; Spangle et al. 2017). These findings showed a rationale for the putative role of PI3Kγ affecting epigenetic regulations, followed by metabolic changes and thus inducing trained sensitization and tolerance.

Pathological events trigger the activation of microglial cells transforming them to active phagocytes, and thus serving as key cells for the maintaining and protecting the neurologic network in CNS from invading pathogens (Pocock and Kettenmann 2007; Kettenmann et al. 2013). Published work from our group showed that LPS-induced phagocytic activity in microglial cells is mediated by downregulation of cAMP-dependent pathway, tightly mediated by the scaffold activity of PI3Kγ (Schmidt et al. 2013). Our investigation showed that PI3Kγ$^{-/-}$ mice strains displayed decreased phagocytic activity compared to wild-type genotype characterized by increased levels of cAMP production (Fig. 22), indicating lipid kinase-independent control of cAMP by PI3Kγ as key mediator of microglial phagocytosis *in vitro* as well as *in vivo* (Fig. 21, 23). Furthermore, wild-type cells primed with high-dose LPS inducing tolerance showed increased phagocytic activity compared to unprimed control *in vitro* and *in vivo*. Comparable findings were reported, where microglial cells *in vivo* expressed increasing markers for the microglial phagocytosis (Wendeln et al. 2018).

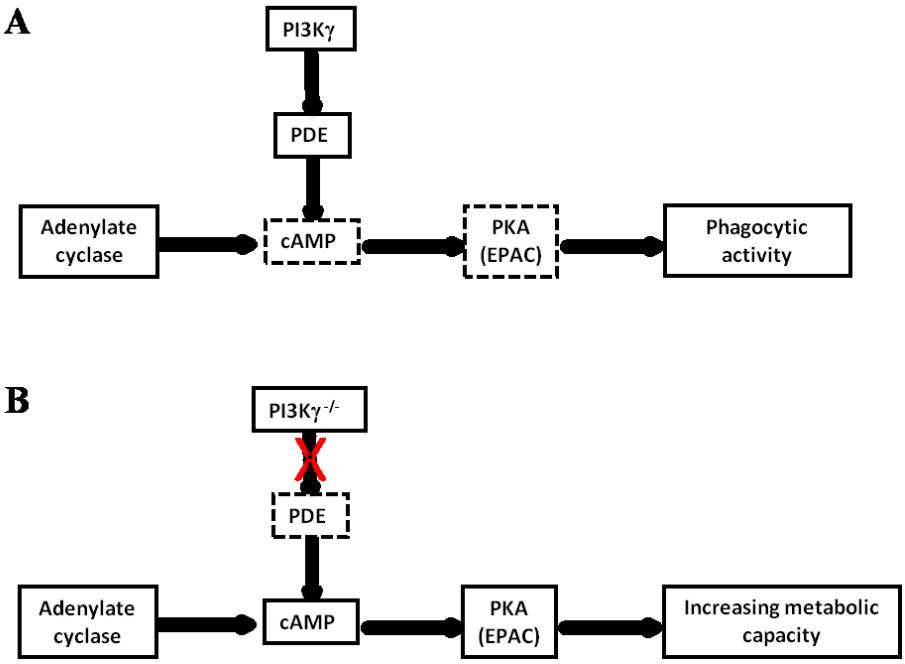

Figure 27: Schematic overview of the lipid kinase-independent function of PI3Kγ regulating phagocytic activity and metabolic changes in microglial cells.
(A) PI3Kγ mediates the activation (→) of PDE, triggering degradation of cAMP, thus leading to decreased levels of cAMP resulting with increased phagocytic activity (Schmidt et al. 2013). In contrast, (B) PI3Kγ$^{-/-}$ (knock-out) is unable (X) to induce the activation of PDE, leading to increased levels of cAMP resulting with increased metabolic activity.

Taken together, our study revealed the control of cAMP levels mediated by PI3Kγ scaffold function, as crucial mediator for regulation of microglial phagocytosis and metabolic activities during trained sensitization and tolerance.

In addition, experiments performed on the 3 month old mice strains (wild-type, PI3Kγ$^{-/-}$ and PI3Kγ$^{KD/KD}$) revealed that mice primed with low-dose as well high-dose of LPS showed after subsequent injection with LPS similar regulation of the cytokine profile (TNF-α and IL-6) in blood plasma and brain tissue as shown *in vitro* in microglial cells, mediated by the lipid kinase-dependent activity of PI3Kγ (Fig. 24). Interestingly, brain tissue cytokine production (TNF-α, IL-6) after subsequent injection by LPS showed increased amounts compared to previous studies done with single injection LPS, expressing a phenotype complementary to trained sensitization, whereas blood plasma cytokines (TNF-α, IL-6) showed decreased tendency compared to single injection studies displaying a phenotype toward tolerance response (Ndongson-Dongmo et al. 2015; Ndongson-Dongmo et al. 2018, under revision). The importance of low- and high-dose

LPS in the brain was reported to affect the occurrence and severity of the neurodegenerative disorders in the mice, where low-dose LPS displayed increased production of pro-inflammatory cytokines resulting with increased accumulation of β-amyloid plaques, whereas repeated injection by high-dose LPS displayed reduced levels of pro-inflammatory cytokines characterized by reduced amounts of β-amyloid plaque accumulation (Wendeln et al. 2018).

Taken together, our results till now revealed the pathogen dose as key determinant for the induction of trained sensitization by low-dose PAMPs (LPS, β-glucan) and tolerance by high-dose PAMPs (LPS, β-glucan) in microglial cells. Metabolic changes play a pivotal role in the induction of both of adaptive responses, characterized mainly also with different attitudes of cellular functions, especially phagocytosis, tightly mediated by the lipid kinase-dependent (e.g. cytokine production) and -independent (metabolic changes and phagocytosis) activities of PI3Kγ. Exhibition of putative effects of trained sensitization and tolerance affecting different neurodegenerative disorders remains so far unknown and represents a major challenge for the future research.

VI. Conclusions

Our findings characterize the critical importance of the sensing dose of PAMPs (LPS, β-glucan) for induction of trained sensitization - by low priming dosages - and tolerance by high-dose priming of microglial cells. Furthermore, our data report that lipid kinase-dependent and -independent (scaffold) activities of PI3Kγ mediate the cytokine profile (TNF-α, IL-6 and IL-10) by the activation or inhibition of the NFκB pathway, metabolic changes (glycolysis and oxidative phosphorylation) and cellular functions such as phagocytosis, affecting the induction of trained sensitization and tolerance response in microglial cells.

Therefore, PI3Kγ was identified to be a key mediator that promotes the dose-dependent induction of memory-like effects imposed by transcriptional modifications and resulting metabolic rearrangements in microglial cells.

References

Aguzzi A, Barres BA, Bennett ML (2013) Microglia: Scapegoat, Saboteur, or Something Else? Science (80-) 339:156–161

Akinleye A, Avvaru P, Furqan M, Song Y, Liu D (2013) Phosphatidylinositol 3-kinase (PI3K) inhibitors as cancer therapeutics. J Hematol Oncol 6:88

Akira S, Takeda K (2004) Toll-like receptor signalling. Nat Rev Immunol 4:499–511

Akira S, Uematsu S, Takeuchi O (2006) Pathogen recognition and innate immunity. Cell 124:783–801

Amor S, Puentes F, Baker D, Valk P van der (2010) Inflammation in neurodegenerative diseases. Immunology 129:154

Arts RJW et al. (2016a) Immunometabolic Pathways in BCG-Induced Trained Immunity. Cell Rep 17:2562–2571

Arts RJW et al. (2016b) Glutaminolysis and Fumarate Accumulation Integrate Immunometabolic and Epigenetic Programs in Trained Immunity. Cell Metab 24:807–819

Avdic S, Cao JZ, McSharry BP, Clancy LE, Brown R, Steain M, Gottlieb DJ, Abendroth A, Slobedman B (2013) Human Cytomegalovirus Interleukin-10 Polarizes Monocytes toward a Deactivated M2c Phenotype To Repress Host Immune Responses. J Virol 87:10273–82

Bader AG, Kang S, Zhao L, Vogt PK (2005) Oncogenic PI3K deregulates transcription and translation. Nat Rev Cancer 5:921–929

Bauer M, Weis S, Netea MG, Wetzker R (2018) Remembering Pathogen Dose: Long-Term Adaptation in Innate Immunity. Trends Immunol 39:438–445

Becattini B, Marone R, Zani F, Arsenijevic D, Seydoux J, Montani J-P, Dulloo AG, Thorens B, Preitner F, Wymann MP, Solinas G (2011) PI3K within a nonhematopoietic cell type negatively regulates diet-induced thermogenesis and promotes obesity and insulin resistance. Proc Natl Acad Sci 108:E854–E863

Becker L, Gharib SA, Irwin AD, Wijsman E, Vaisar T, Oram JF, Heinecke JW (2010) A Macrophage Sterol-Responsive Network Linked to Atherogenesis. Cell Metab 11:125–135

Beeson P (1947) Tolerance To Bacterial Pyrogens : Role of the Reticulo-Endothelial System. J Exp Med 86:39–44

Bekkering S, Joosten LABB, Meer JWM van der, Netea MG, Riksen NP, Van Der Meer JWM, Netea MG, Riksen NP (2013) Trained innate immunity and atherosclerosis. Curr Opin Lipidol 24:487–492

Biswas SK, Lopez-Collazo E (2009) Endotoxin tolerance: new mechanisms, molecules and clinical significance. Trends Immunol 30:475–487

Breasson L, Becattini B, Sardi C, Molinaro A, Zani F, Marone R, Botindari F, Bousquenaud M, Ruegg C, Wymann MP, Solinas G (2017) PI3Kg activity in leukocytes promotes adipose tissue inflammation and early-onset insulin resistance during obesity. Sci Signal 10:eaaf2969

Breasson L, Sardi C, Becattini B, Zani F, Solinas G (2018) PI3Kg ablation does not promote diabetes in db/db mice, but improves insulin sensitivity and reduces pancreatic b-cell apoptosis. FASEB J 32:319–329

Buffen K et al. (2014) Autophagy Controls BCG-Induced Trained Immunity and the Response to Intravesical BCG Therapy for Bladder Cancer. PLoS Pathog 10:e1004485

Butovsky O, Weiner HL (2018) Microglial signatures and their role in health and disease. Nat Rev Neurosci 19:622–635

Calabrese EJ, Baldwin LA (2001) Hormesis: U-shaped dose responses and their centrality in toxicology. Trends Pharmacol Sci 22:285–291

Cavaillon J-M, Adib-Conquy M (2006) Bench-to-bedside review: endotoxin tolerance as a model of leukocyte reprogramming in sepsis. Crit Care 10:233

Chen K, Geng S, Yuan R, Diao N, Upchurch Z, Li L (2015) Super-low Dose Endotoxin Pre-conditioning Exacerbates Sepsis Mortality. EBioMedicine 2:324–333

Cheng S-C et al. (2014) mTOR- and HIF-1 -mediated aerobic glycolysis as metabolic basis for trained immunity. Science (80-) 345:1250684

Chu C-H, Wang S, Li C-L, Chen S-H, Hu C-F, Chung Y-L, Chen S-L, Wang Q, Lu R-B, Gao H-M, Hong J-S (2016) Neurons and astroglia govern microglial endotoxin tolerance through macrophage colony-stimulating factor receptor-mediated ERK1/2 signals. Brain Behav Immun 55:260–272

Civciristov S, Ellisdon AM, Suderman R, Pon CK, Evans BA, Kleifeld O, Charlton SJ, Hlavacek WS, Canals M, Halls ML (2018) Preassembled GPCR signaling complexes mediate distinct cellular responses to ultralow ligand concentrations. Sci Signal 11:eaan1188

Colonna M, Butovsky O (2017) Microglia Function in the Central Nervous System During Health and Neurodegeneration. Annu Rev Immunol 35:441–468

Costa C, Hirsch E (2010) More than just kinases: The scaffolding function of PI3K. Curr Top Microbiol Immunol 346:171–181

Crișan TO, Netea MG, Joosten LAB (2016) Innate immune memory: Implications for host responses to damage-associated molecular patterns. Eur J Immunol 46:817–828

Davis BM, Salinas-Navarro M, Cordeiro MF, Moons L, De Groef L, Groef L De (2017) Characterizing microglia activation: A spatial statistics approach to maximize information extraction. Sci Rep 7:1576

De Lima TM, Sampaio SC, Petroni R, Brigatte P, Velasco IT, Soriano FG (2014) Phagocytic

activity of LPS tolerant macrophages. Mol Immunol 60:8–13

Devereaux K, Dall'Armi C, Alcazar-Roman A, Ogasawara Y, Zhou X, Wang F, Yamamoto A, de Camilli P, Di Paolo G (2013) Regulation of Mammalian Autophagy by Class II and III PI 3-Kinases through PI3P Synthesis. PLoS One 8:e76405

Dheen ST, Kaur C, Ling E-A (2007) Microglial activation and its implications in the brain diseases. Curr Med Chem 14:1189–97

Dinarello CA (1992) Cytokines: agents provocateurs in hemodialysis? Kidney Int 41:683–94

Domínguez-Andrés J et al. (2018) The Itaconate Pathway Is a Central Regulatory Node Linking Innate Immune Tolerance and Trained Immunity. Cell Metab pii:S1550-4131(18)30568–0

Doxaki C, Kampranis SC, Eliopoulos AG, Spilianakis C, Tsatsanis C (2015) Coordinated Regulation of miR-155 and miR-146a Genes during Induction of Endotoxin Tolerance in Macrophages. J Immunol 195:5750–5761

Dubois RN (2015) The Jeremiah Metzger Lecture: Inflammation, Immune Modulators, and Chronic Disease. Trans Am Clin Climatol Assoc 126:230–6

Dziarski R, Gupta D (2000) Role of MD-2 in TLR2- and TLR4-mediated recognition of Gram-negative and Gram-positive bacteria and activation of chemokine genes. J Endotoxin Res 6:401–5

Falasca M, Maffucci T (2012) Regulation and cellular functions of class II phosphoinositide 3-kinases. Biochem J 443:587–601

Fensterheim BA, Guo Y, Sherwood ER, Bohannon JK (2017) The Cytokine Response to Lipopolysaccharide Does Not Predict the Host Response to Infection. J Immunol 198:3264–3273

Foster SL, Hargreaves DC, Medzhitov R (2007) Gene-specific control of inflammation by TLR-induced chromatin modifications. Nature 451:972–8

Frister A, Schmidt C, Schneble N, Brodhun M, Gonnert F a., Bauer M, Hirsch E, Müller JP, Wetzker R, Bauer R (2014) Phosphoinositide 3-Kinase γ Affects LPS-Induced Disturbance of Blood–Brain Barrier Via Lipid Kinase-Independent Control of cAMP in Microglial Cells. NeuroMolecular Med 16:704–713

Fruman DA, Chiu H, Hopkins BD, Bagrodia S, Cantley LC, Abraham RT (2017) The PI3K Pathway in Human Disease. Cell 170:605–635

Gaidarov I, Smith MEK, Domin J, Keen JH (2001) The class II phosphoinositide 3-kinase C2α is activated by clathrin and regulates clathrin-mediated membrane trafficking. Mol Cell 7:443–9

Gantner BN, Singh H (2007) Short-term memory. Nature 447:916–917

Garly M-LL, Martins CL, Balé C, Baldé MA, Hedegaard KL, Gustafson P, Lisse IM, Whittle HC, Aaby P (2003) BCG scar and positive tuberculin reaction associated with reduced child

mortality in West Africa: A non-specific beneficial effect of BCG? Vaccine 21:2782–90

Gasparini C, Foxwell BM, Feldmann M (2013) RelB/p50 regulates TNF production in LPS-stimulated dendritic cells and macrophages. Cytokine 61:736–740

Ghosh S, Karin M (2002) Missing Pieces in the NF-κB Puzzle. Cell 109:S81–S96

Gioannini TL, Teghanemt A, Zhang D, Coussens NP, Dockstader W, Ramaswamy S, Weiss JP (2004) Isolation of an endotoxin-MD-2 complex that produces Toll-like receptor 4-dependent cell activation at picomolar concentrations. Proc Natl Acad Sci 101:4186–4191

Glass CK, Saijo K, Winner B, Marchetto MC, Gage FH (2010) Mechanisms Underlying Inflammation in Neurodegeneration. Cell 140:918–934

Godbout JP, Chen J, Abraham J, Richwine AF, Berg BM, Kelley KW, Johnson RW (2005) Exaggerated neuroinflammation and sickness behavior in aged mice following activation of the peripheral innate immune system. FASEB J 19:1329–1331

Grabert K, Michoel T, Karavolos MH, Clohisey S, Baillie JK, Stevens MP, Freeman TC, Summers KM, McColl BW (2016) Microglial brain region−dependent diversity and selective regional sensitivities to aging. Nat Neurosci 19:504–516

Guillot L, Le Goffic R, Bloch S, Escriou N, Akira S, Chignard M, Si-Tahar M (2005) Involvement of toll-like receptor 3 in the immune response of lung epithelial cells to double-stranded RNA and influenza A virus. J Biol Chem 280:5571–80

Ha JS, Kwon K-SS, Park SS (2013) PI3Kγ contributes to MEK1/2 activation in oxidative glutamate toxicity via PDK1. J Neurochem 127:139–48

Habib P, Slowik A, Zendedel A, Johann S, Dang J, Beyer C (2014) Regulation of hypoxia-induced inflammatory responses and M1-M2 phenotype switch of primary rat microglia by Sex steroids. J Mol Neurosci 52:277–85

Häcker H, Redecke V, Blagoev B, Kratchmarova I, Hsu LC, Wang GG, Kamps MP, Raz E, Wagner H, Häcker G, Mann M, Karin M (2006) Specificity in Toll-like receptor signalling through distinct effector functions of TRAF3 and TRAF6. Nature 439:204–7

Hanisch U-K (2002) Microglia as a source and target of cytokines. Glia 40:140–155

Harry GJ, Kraft AD (2012) Microglia in the developing brain: A potential target with lifetime effects. Neurotoxicology 33:191–206

Hawkins PT, Anderson KE, Davidson K, Stephens LR (2006) Signalling through Class I PI3Ks in mammalian cells. Biochem Soc Trans 34:647–662

Hawkins PT, Stephens LR (2007) PI3Kγ is a key regulator of inflammatory responses and cardiovascular homeostasis. Science (80-) 318:64–66

Hoesel B, Schmid JA (2013) The complexity of NF-κB signaling in inflammation and cancer. Mol Cancer 12:86

Hooper MJ, Wang J, Browning R, Ash JD (2018) Damage-associated molecular pattern recognition is required for induction of retinal neuroprotective pathways in a sex-dependent manner. Sci Rep 8:9115

Ifrim DC, Quintin J, Joosten LAB, Jacobs C, Jansen T, Jacobs L, Gow NAR, Williams DL, van der Meer JWM, Netea MG (2014) Trained Immunity or Tolerance: Opposing Functional Programs Induced in Human Monocytes after Engagement of Various Pattern Recognition Receptors. Clin Vaccine Immunol 21:534–545

Jacque E, Tchenio T, Piton G, Romeo P-HP-H, Baud V (2005) RelA repression of RelB activity induces selective gene activation downstream of TNF receptors. Proc Natl Acad Sci U S A 102:14635–14640

Janeway CA, Medzhitov R (2002) Innate immune recognition. Annu Rev Immunol 20:197–216

Jeon S-MM (2016) Regulation and function of AMPK in physiology and diseases. Exp Mol Med 48:e245

Jha AK, Huang SCC, Sergushichev A, Lampropoulou V, Ivanova Y, Loginicheva E, Chmielewski K, Stewart KM, Ashall J, Everts B, Pearce EJ, Driggers EM, Artyomov MN (2015) Network integration of parallel metabolic and transcriptional data reveals metabolic modules that regulate macrophage polarization. Immunity 42:419–30

Jin R, Song Z, Yu S, Piazza A, Nanda A, Penninger JM, Granger DN, Li G (2011) Phosphatidylinositol-3-kinase gamma plays a central role in blood-brain barrier dysfunction in acute experimental stroke. Stroke 42:2033–2044

Jin R, Yu S, Song Z, Quillin JW, Deasis DP, Penninger JM, Nanda A, Granger DN, Li G (2010) Phosphoinositide 3-kinase-gamma expression is upregulated in brain microglia and contributes to ischemia-induced microglial activation in acute experimental stroke. Biochem Biophys Res Commun 399:458–464

Kaneda MM et al. (2016) PI3Kγ 3 is a molecular switch that controls immune suppression. Nature 539:437–442

Kawai T, Takeuchi O, Fujita T, Inoue J -i., Muhlradt PF, Sato S, Hoshino K, Akira S, Mühlradt PF, Sato S, Hoshino K, Akira S (2001) Lipopolysaccharide Stimulates the MyD88-Independent Pathway and Results in Activation of IFN-Regulatory Factor 3 and the Expression of a Subset of Lipopolysaccharide-Inducible Genes. J Immunol 167:5887–94

Kettenmann H, Hanisch U, Noda M, Verkhratsky A (2011) Physiology of Microglia. Physiol Rev 91:461–553

Kettenmann H, Kirchhoff F, Verkhratsky A (2013) Microglia: New Roles for the Synaptic Stripper. Neuron 77:10–18

Kigerl KA, de Rivero Vaccari JP, Dietrich WD, Popovich PG, Keane RW (2014) Pattern recognition receptors and central nervous system repair. Exp Neurol 258:5–16

Kim CC, Nakamura MC, Hsieh CL (2016) Brain trauma elicits non-canonical macrophage

activation states. J Neuroinflammation 13:117

Kim J, Kwak HJ, Cha JY, Jeong YS, Rhee SD, Kim KR, Cheon HG (2014) Metformin suppresses lipopolysaccharide (LPS)-induced inflammatory response in murine macrophages via Activating Transcription Factor-3 (ATF-3) induction. J Biol Chem 289:23246–55

Kim YS, Joh TH (2006) Microglia, major player in the brain inflammation: Their roles in the pathogenesis of Parkinson's disease. Exp Mol Med 38:333–347

Kleinnijenhuis J, Quintin J, Preijers F, Joosten LAB, Ifrim DC, Saeed S, Jacobs C, van Loenhout J, de Jong D, Stunnenberg HG, Xavier RJ, van der Meer JWM, van Crevel R, Netea MG (2012) Bacille Calmette-Guerin induces NOD2-dependent nonspecific protection from reinfection via epigenetic reprogramming of monocytes. Proc Natl Acad Sci 109:17537–17542

Kobayashi K, Imagama S, Ohgomori T, Hirano K, Uchimura K, Sakamoto K, Hirakawa A, Takeuchi H, Suzumura A, Ishiguro N, Kadomatsu K (2013) Minocycline selectively inhibits M1 polarization of microglia. Cell Death Dis 4:e525

König C, Gavrilova-Ruch O, von Banchet GS, Bauer R, Grün M, Hirsch E, Rubio I, Schulz S, Heinemann SH, Schaible HG, Wetzker R (2010) Modulation of μ-opioid receptor desensitization in peripheral sensory neurons by phosphoinositide 3-kinase γ. Neuroscience 169:449–454

Kwon H-J, Choi G-E, Ryu S, Kwon SJ, Kim SC, Booth C, Nichols KE, Kim HS (2016) Stepwise phosphorylation of p65 promotes NF-κB activation and NK cell responses during target cell recognition. Nat Commun 7:11686

Landau G, Bercovich Z, Park MH, Kahana C (2010) The role of polyamines in supporting growth of mammalian cells is mediated through their requirement for translation initiation and elongation. J Biol Chem 285:

Lawrence T (2009) The nuclear factor NF-kappaB pathway in inflammation. Cold Spring Harb Perspect Biol 1:a001651

Lawson LJ, Perry VH, Dri P, Gordon S (1990) Heterogeneity in the distribution and morphology of microglia in the normal adult mouse brain. Neuroscience 39:151–70

Lin C-Y, Chen S-H, Kou G-H, Kuo C-M (1999) An enzymatic microassay for lactate concentration in blood and hemolymph. Acta Zool taiwanica 10:91–101

Liu P, Cheng H, Roberts TM, Zhao JJ (2009) Targeting the phosphoinositide 3-kinase (PI3K) pathway in cancer. Nat Rev Drug Discov 8:627–644

Liu T, Zhang L, Joo D, Sun S-C (2017) NF-κB signaling in inflammation. Signal Transduct Target Ther 2:17023

Liu X, Qin Y, Dai A, Zhang Y, Xue H, Ni H, Han L, Zhu L, Yuan D, Tao T, Cao M (2016) SMAD4 is Involved in the Development of Endotoxin Tolerance in Microglia. Cell Mol

Neurobiol 36:777–88

Lull ME, Block ML (2010) Microglial Activation and Chronic Neurodegeneration. Neurotherapeutics 7:354–365

Luo L, Wall AA, Yeo JC, Condon ND, Norwood SJ, Schoenwaelder S, Chen KW, Jackson S, Jenkins BJ, Hartland EL, Schroder K, Collins BM, Sweet MJ, Stow JL (2014) Rab8a interacts directly with PI3Kγ to modulate TLR4-driven PI3K and mTOR signalling. Nat Commun 5:4407

Luo X-G, Ding J-Q, Chen S-D (2010) Microglia in the aging brain: relevance to neurodegeneration. Mol Neurodegener 5:12

Malek M et al. (2017) PTEN Regulates PI(3,4)P2Signaling Downstream of Class I PI3K. Mol Cell 68:566–580.e10

Martini M, De Santis MC, Braccini L, Gulluni F, Hirsch E (2014) PI3K/AKT signaling pathway and cancer: an updated review. Ann Med 46:372–83

Mavrommati I, Cisse O, Falasca M, Maffucci T (2016) Novel roles for class II Phosphoinositide 3-Kinase C2β in signalling pathways involved in prostate cancer cell invasion. Sci Rep 6:23277

Medzhitov R (2007) Recognition of microorganisms and activation of the immune response. Nature 449:819–826

Morganti JM, Riparip L-K, Rosi S (2016) Call Off the Dog(ma): M1/M2 Polarization Is Concurrent following Traumatic Brain Injury. PLoS One 11:e0148001

Morin M, Schindler R, Wakabayashi G, Daumy G, Dinarello CA, Gelfand JA Picogram concentrations of endotoxin stimulate synthesis of IL-1 beta and TNF alpha by human peripheral blood mononuclear cells exposed to recombinant human C5a. Eur Cytokine Netw 2:27–30

Murray PJ, Wynn TA (2011) Protective and pathogenic functions of macrophage subsets. Nat Rev Immunol 11:723–737

Ndongson-Dongmo B, Heller R, Hoyer D, Brodhun M, Bauer M, Winning J, Hirsch E, Wetzker R, Schlattmann P, Bauer R (2015) Phosphoinositide 3-kinase gamma controls inflammation-induced myocardial depression via sequential cAMP and iNOS signalling. Cardiovasc Res 108:243–253

Netea MG, Joosten LAB, Latz E, Mills KHG, Natoli G, Stunnenberg HG, ONeill LAJ, Xavier RJ (2016) Trained immunity: A program of innate immune memory in health and disease. Science (80-) 352:aaf1098

Netea MG, Quintin J, van der Meer JWM (2011) Trained Immunity: A Memory for Innate Host Defense. Cell Host Microbe 9:355–361

Netea MG, van der Meer JWM (2017) Trained Immunity: An Ancient Way of Remembering.

Cell Host Microbe 21:297–300

Nhu QM, Shirey KA, Pennini ME, Stiltz J, Vogel SN (2012) Proteinase-activated receptor 2 activation promotes an anti-inflammatory and alternatively activated phenotype in LPS-stimulated murine macrophages. Innate Immun 18:193–203

Nikodemova M, Small AL, Kimyon RS, Watters JJ (2016) Age-dependent differences in microglial responses to systemic inflammation are evident as early as middle age. Physiol Genomics 48:336–44

Nimmerjahn A, Kirchhoff F, Helmchen F (2005) Neuroscience: Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo. Science (80-) 308:1314–1318

Nomura F, Akashi S, Sakao Y, Sato S, Kawai T, Matsumoto M, Nakanishi K, Kimoto M, Miyake K, Takeda K, Akira S (2000) Cutting Edge: Endotoxin Tolerance in Mouse Peritoneal Macrophages Correlates with Down-Regulation of Surface Toll-Like Receptor 4 Expression. J Immunol 164:3476–9

Norata GD (2018) Trained immunity and cardiovascular disease: is it time for translation to humans? Cardiovasc Res 114:e41–e42

Novakovic B et al. (2016) β-Glucan Reverses the Epigenetic State of LPS-Induced Immunological Tolerance. Cell 167:1354–1368.e14

Okkenhaug K, Vanhaesebroeck B (2003) PI3K in lymphocyte development, differentiation and activation. Nat Rev Immunol 3:317–330

Orihuela R, McPherson CA, Harry GJ (2016) Microglial M1/M2 polarization and metabolic states. Br J Pharmacol 173:649–665

Paramo T, Tomasio SM, Irvine KL, Bryant CE, Bond PJ (2016) Energetics of Endotoxin Recognition in the Toll-Like Receptor 4 Innate Immune Response. Sci Rep 5:17997

Patrucco E et al. (2004) PI3Kgamma modulates the cardiac response to chronic pressure overload by distinct kinase-dependent and -independent effects. Cell 118:375–87

Paust S, Gill HS, Wang B-ZZ, Flynn MP, Moseman EA, Senman B, Szczepanik M, Telenti A, Askenase PW, Compans RW, von Andrian UH (2010) Critical role for the chemokine receptor CXCR6 in NK cell-mediated antigen-specific memory of haptens and viruses. Nat Immunol 11:1127–1135

Pena OM, Pistolic J, Raj D, Fjell CD, Hancock REW (2011) Endotoxin Tolerance Represents a Distinctive State of Alternative Polarization (M2) in Human Mononuclear Cells. J Immunol 186:7243–7254

Perino A et al. (2014) Combined inhibition of PI3K and PI3K reduces fat mass by enhancing -MSH-dependent sympathetic drive. Sci Signal 7:ra110

Pesce JT, Ramalingam TR, Mentink-Kane MM, Wilson MS, Kasmi KCE, Smith AM, Thompson RW, Cheever AW, Murray PJ, Wynn TA (2009) Arginase-1-expressing macrophages

suppress Th2 cytokine-driven inflammation and fibrosis. PLoS Pathog 5:e1000371

Plato A, Hardison SE, Brown GD (2015) Pattern recognition receptors in antifungal immunity. Semin Immunopathol 37:97–106

Pocock JM, Kettenmann H (2007) Neurotransmitter receptors on microglia. Trends Neurosci 30:527–535

Poplutz M, Levikova M, Lüscher-Firzlaff J, Lesina M, Algül H, Lüscher B, Huber M (2017) Endotoxin tolerance in mast cells, its consequences for IgE-mediated signalling, and the effects of BCL3 deficiency. Sci Rep 7:4534

Quintin J et al. (2012) Candida albicans infection affords protection against reinfection via functional reprogramming of monocytes. Cell Host Microbe 12:223–232

Ransohoff RM (2016) A polarizing question: Do M1 and M2 microglia exist. Nat Neurosci 19:987–991

Reiling JH, Sabatini DM (2006) Stress and mTORture signaling. Oncogene 25:6373–6383

Rivera A, Siracusa MC, Yap GS, Gause WC (2016) Innate cell communication kick-starts pathogen-specific immunity. Nat Immunol 17:356–363

Roy A, Jana A, Yatish K, Freidt MB, Fung YK, Martinson JA, Pahan K (2008) Reactive oxygen species up-regulate CD11b in microglia via nitric oxide: Implications for neurodegenerative diseases. Free Radic Biol Med 45:686–99

Rückle T, Schwarz MK, Rommel C (2006) PI3Kgamma inhibition: towards an “aspirin of the 21st century”? Nat Rev Drug Discov 5:903–918

Saeed S et al. (2014) Epigenetic programming of monocyte-to-macrophage differentiation and trained innate immunity. Science (80-) 345:1251086–1251086

Saijo K, Glass CK (2011) Microglial cell origin and phenotypes in health and disease. Nat Rev Immunol 11:775–787

Salam AP, Borsini A, Zunszain PA (2018) Trained innate immunity: A salient factor in the pathogenesis of neuroimmune psychiatric disorders. Mol Psychiatry 23:170–176

Santiago AR, Bernardino L, Agudo-Barriuso M, Gonçalves J (2017) Microglia in Health and Disease: A Double-Edged Sword. Mediators Inflamm 2017:1–2

Schaafsma W et al. (2015) Long-lasting pro-inflammatory suppression of microglia by LPS-preconditioning is mediated by RelB-dependent epigenetic silencing. Brain Behav Immun 48:205–221

Schmidt C, Frahm C, Schneble N, Müller JP, Brodhun M, Franco I, Witte OW, Hirsch E, Wetzker R, Bauer R (2016) Phosphoinositide 3-Kinase γ Restrains Neurotoxic Effects of Microglia After Focal Brain Ischemia. Mol Neurobiol 53:5468–79

Schmidt C, Schneble N, Müller JP, Bauer R, Perino a, Marone R, Rybalkin SD, Wymann MP,

Hirsch E, Wetzker R (2013) Phosphoinositide 3-kinase γ mediates microglial phagocytosis via lipid kinase-independent control of cAMP. Neuroscience 233:44–53

Schmittgen TD, Livak KJ (2008) Analyzing real-time PCR data by the comparative CT method. Nat Protoc 3:1101–1108

Schneble N, Schmidt C, Bauer R, Müller JP, Monajembashi S, Wetzker R (2017) Phosphoinositide 3-kinase γ ties chemoattractant- and adrenergic control of microglial motility. Mol Cell Neurosci 78:1–8

Schrum JE, Crabtree JN, Dobbs KR, Kiritsy MC, Reed GW, Gazzinelli RT, Netea MG, Kazura JW, Dent AE, Fitzgerald KA, Golenbock DT (2018) Cutting Edge: Plasmodium falciparum Induces Trained Innate Immunity. J Immunol 200:1243–1248

Shemer A, Erny D, Jung S, Prinz M (2015) Microglia Plasticity During Health and Disease: An Immunological Perspective. Trends Immunol 36:614–624

Sly LM, Rauh MJ, Kalesnikoff J, Song CH, Krystal G (2004) LPS-induced upregulation of SHIP is essential for endotoxin tolerance. Immunity 21:227–239

Soares MP, Gozzelino R, Weis S (2014) Tissue damage control in disease tolerance. Trends Immunol 35:483–494

Spangle JM, Roberts TM, Zhao JJ (2017) The emerging role of PI3K/AKT-mediated epigenetic regulation in cancer. Biochim Biophys acta Rev cancer 1868:123–131

Stevens WBC, Netea MG, Kater AP, van der Velden WJFM (2016) "Trained immunity": consequences for lymphoid malignancies. Haematologica 101:1460–1468

Streit WJ (2002) Microglia as neuroprotective, immunocompetent cells of the CNS. Glia 40:133–139

Sun H-N, Kim S-KS-U, Lee M-S, Kim S-KS-U, Kim J-M, Yim M, Yu D-Y, Lee D-S (2008) Nicotinamide Adenine Dinucleotide Phosphate (NADPH) Oxidase-Dependent Activation of Phosphoinositide 3-Kinase and p38 Mitogen-Activated Protein Kinase Signal Pathways Is Required for Lipopolysaccharide-Induced Microglial Phagocytosis. Biol Pharm Bull 31:1711–5

Sun JC, Beilke JN, Lanier LL (2009) Adaptive immune features of natural killer cells. Nature 457:557–561

Takeuchi O, Akira S (2010) Pattern Recognition Receptors and Inflammation. Cell 140:805–820

Talge NM, Neal C, Glover V, Early Stress TR and PSNF and NE on C and AMH (2007) Antenatal maternal stress and long-term effects on child neurodevelopment: How and why? J Child Psychol Psychiatry Allied Discip 48:245–261

Tang Y, Le W (2016) Differential Roles of M1 and M2 Microglia in Neurodegenerative Diseases. Mol Neurobiol 53:1181–1194

Taylor RA et al. (2017) TGF-β1 modulates microglial phenotype and promotes recovery after

intracerebral hemorrhage. J Clin Invest 127:280–292

Thorpe LM, Yuzugullu H, Zhao JJ (2015) PI3K in cancer: Divergent roles of isoforms, modes of activation and therapeutic targeting. Nat Rev Cancer 15:7–24

Toska E, Osmanbeyoglu HU, Castel P, Chan C, Hendrickson RC, Elkabets M, Dickler MN, Scaltriti M, Leslie CS, Armstrong SA, Baselga J (2017) PI3K pathway regulates ER-dependent transcription in breast cancer through the epigenetic regulator KMT2D. Science 355:1324–1330

Vadas O, Dbouk HA, Shymanets A, Perisic O, Burke JE, Abi Saab WF, Khalil BD, Harteneck C, Bresnick AR, Nurnberg B, Backer JM, Williams RL (2013) Molecular determinants of PI3K -mediated activation downstream of G-protein-coupled receptors (GPCRs). Proc Natl Acad Sci 110:18862–18867

Van den Bossche J et al. (2016) Mitochondrial Dysfunction Prevents Repolarization of Inflammatory Macrophages. Cell Rep 17:684–696

van der Meer JWM, Joosten LAB, Riksen N, Netea MG (2015) Trained immunity: A smart way to enhance innate immune defence. Mol Immunol 68:40–44

Walter L, Neumann H (2009) Role of microglia in neuronal degeneration and regeneration. Semin Immunopathol 31:513–525

Wegiel B, Hauser CJ, Otterbein LE (2015) Heme as a danger molecule in pathogen recognition. Free Radic Biol Med 89:651–661

Weichhart T, Hengstschlager M, Linke M (2015) Regulation of innate immune cell function by mTOR. Nat Rev Immunol 15:599–614

Wen H, Dou Y, Hogaboam CM, Kunkel SL (2008) Epigenetic regulation of dendritic cell-derived interleukin-12 facilitates immunosuppression after a severe innate immune response. Blood 111:1797–1804

Wendeln A-C et al. (2018) Innate immune memory in the brain shapes neurological disease hallmarks. Nature 556:332–338

West M a, Heagy W (2002) Endotoxin tolerance: A review. Crit Care Med 30:S64–S73

White CA, McCombe PA, Pender MP (1998) Microglia are more susceptible than macrophages to apoptosis in the central nervous system in experimental autoimmune encephalomyelitis through a mechanism not involving Fas (CD95). Int Immunol 10:935–41

Wilson M, Henderson B (Professor), McNab R (2002) Bacterial disease mechanisms : an introduction to cellular microbiology. Cambridge University Press

Xu H, Chen J, Si X, Chen M, Pei F, Qiu C, Wu J, Guan X (2018) PKR inhibition mediates endotoxin tolerance in macrophages through inactivation of PI3K/AKT signaling. Mol Med Rep 17:8548–8556

Yamamoto M, Sato S, Hemmi H, Hoshino K, Kaisho T, Sanjo H, Takeuchi O, Sugiyama M,

Okabe M, Takeda K, Akira S (2003) Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. Science (80-) 301:640–643

Yancopoulos GD, Alt FW (1985) Developmentally controlled and tissue-specific expression of unrearranged VH gene segments. Cell 40:271–81

Yu JSL, Cui W (2016) Proliferation, survival and metabolism: the role of PI3K/AKT/mTOR signalling in pluripotency and cell fate determination. Development 143:3050–3060

Yuan R, Geng S, Li L (2016) Molecular mechanisms that underlie the dynamic adaptation of innate monocyte memory to varying stimulant strength of TLR ligands. Front Immunol 7:497

Zhang HH, Lipovsky AI, Dibble CC, Sahin M, Manning BD (2006) S6K1 Regulates GSK3 under Conditions of mTOR-Dependent Feedback Inhibition of Akt. Mol Cell 24:185–197

Zhang Y, Kim MS, Jia B, Yan J, Zuniga-Hertz JP, Han C, Cai D (2017) Hypothalamic stem cells control ageing speed partly through exosomal miRNAs. Nature 548:52–57

Zhao W, Qiu Y, Kong D (2017) Class I phosphatidylinositol 3-kinase inhibitors for cancer therapy. Acta Pharm Sin B 7:27–37

Zuo T et al. (2011) Epigenetic silencing mediated through activated PI3K/AKT signaling in breast cancer. Cancer Res 71:1752–62

Curriculum Vitae

Personal Information Full Name	**Trim Lajqi**
Date and Place of birth Address	4th of January 1990, Kosovo Fischergasse 4, 07743 Jena, Germany
E-mail (official) E-mails (personal)	Trim.Lajqi@med.uni-jena.de trim-dark@hotmail.com; trimlajqi049@gmail.com
Education 10/2015 – Present	PhD Student Jena University Hospital, Institute of Molecular Cell Biology, Jena (Germany)
10/2015 – 10/2018	Research Training Group 1715 – from DFG Jena University Hospital, Jena (Germany)
12/2013 – 05/2015	Visiting Researcher Free University of Berlin, Institute of Pharmacy, Berlin (Germany)
10/2008 – 06/2013	Magister of Pharmacy (Master Degree) University of Prishtina, Faculty of Medicine, Prishtina (Kosovo)
Personal Skills Languages	Albanian (mother tongue) English (Fluently) German (Fluently): B2 (Goethe Certificate) and C1 (AKSA Magdeburg)
Computer Skills	Microsoft Office (Word, Excel, Power Point, Outlook, Access) Sigma Plot 12.0 Graph Pad Prism 6.0 and ImageJ
Additional Information Scholarship Winner	Erasmus Mundus – Free University of Berlin (12/2013 – 10/2014) and (11/2014 – 05/2015)
	Travel grant "VIBes in Biosciences – International Symposium for PhD students in Life Sciences" – Ghent, Belgium (27/09 – 29/09/2017)
Conferences (abstracts, proceedings, posters)	Said A, Bock S, **Lajqi T**, Müller G, Weindl G (2015) Chloroquine induces IL-23 release from cutaneous dendritic cells via p38 and subsequently promotes IL-17 production by CD4+ T cells. Naunyn Schmiedebergs Arch Pharmacol 388: S27
	Lajqi T, Bauer R, Stojiljkovic M, Schmeer C, Witte O, Wetzker R (2017) LPS and ß-glucan induce dose-dependent long-term adaptation in microglia. Novel Concepts In Innate Immunity, Tübingen, Germany
	Stojiljkovic M, Schmeer C, **Lajqi T**, Wetzker R, Bauer R, Witte O (2017) Senescence and ageing alter the sensitization/ tolerance response of murine brain microglia after LPS stimulation. Novel Concepts In Innate Immunity, Tübingen, Germany
	Stojiljkovic M, Schmeer C, **Lajqi T**, Wetzker R, Bauer R, Witte O (2017) Comparative phenotypic and functional analysis of senescent microglia *in vitro* and aging microglia in the old murine brain in vivo. XIII European Meeting on Glial

	Cells in Health and Disease, Edinburgh, UK Glia Journal, Issue 1, Pages I-XLII, E429. DOI: 10.1002/glia.23157 **Lajqi T**, Bauer R, Stojiljkovic M, Schmeer C, Witte O, Wetzker R (2017) PAMPs induce long-term and dose-dependent adaptive responses in microglial cells *in vitro*. XIII European Meeting on Glial Cells in Health and Disease, Edinburgh, UK Glia Journal, Issue 1, Pages I-XLII, E429-E430. DOI:10.1002/glia.23157 **Lajqi T**, Wetzker R, Bauer R (2018) Pathogen-associated molecular patterns induce development of training and tolerance in primary microglial cells. Innate Immunity in Host-Pathogen Interactions, EMBL Heidelberg, Germany
Memberships	International Society for Infectious Diseases (ISID) Doctoral Council (DR.FSU) and Senator at the Friedrich-Schiller University of Jena, Germany (10/2017 - 10/2018)
Licenses	FELASA B (animal experiments), Jena, Germany Licensed Pharmacist, Kosovo
Publications Book chapter	Jentho E*, **Lajqi T***, Yang K*, Winkler R*, Stojiljkovic M*, Wetzker R, Bauer M. Pathogen-induced hormetic responses. In: The Science of Hormesis in Health and Longevity (1st Edition), ISI Rattan & M Kyriazi (Eds), Academic Press 2018 *(in press; *shared authorship)*
Publications	Said A, Bock S, **Lajqi T**, Müller G, Weindl G (2014) Chloroquine Promotes IL-17 Production by CD4+ T Cells via p38-Dependent IL-23 Release by Monocyte-Derived Langerhans-like Cells. J Immunol 193: 6135-6143. DOI: 10.4049/jimmunol.1303276 **Lajqi T***, Stojiljkovic M*, Wetzker R (2018) Restraining ageing with toxins. Journal of Biogerontology *(under revision) *shared authorship* Ndogson-Dongmo B, Lang GP, Mece O, Hechaichi N, **Lajqi T**, Hoyer D, Brodhun M, Heller R, Wetzker R, Levy FO, Bauer R (2018) Neutral ambient temperature ameliorates SIRS-induced cardiac autonomic dysregulation and myocardial dysfunction in endotoxemic mice. J Basic Research in Cardiology *(under revision)* Lang GP, Ndogson-Dongmo B, **Lajqi T**, Brodhun M, Marx C, Han Y, Wang ZQ, Wetzker R, Bauer R (2018) Reduced ambient temperature enhances inflammation-induced encephalopathy in endotoxemic mice – role of PI3Kγ. Journal of Neuroinflammation *(submitted)* **Lajqi T***, Stojiljkovic M*, Bauer R, Schmeer C (2018) Impact of aging and pathogen dose on immune adaptive responses in murine brain microglia. *(under submission) *shared authorship* **Lajqi T**, Lang GP, Marx C, Wang ZQ, Wetzker R, Bauer R (2018) PI3Kγ – a key regulator of trained immunity and tolerance development in microglial cells. *(in prep)*

Acknowledgements

I would like to express my sincere gratitude to my advisor Prof. Dr. Reinhard Bauer for the continuous support of my PhD study guiding me in every single step during these years and showing me real meaning of being a scientist with great passion for science. You have been a friend, a parent and a guide supporting me with trainings, advices, knowledge and opening me new perspectives for my future. With your patience, motivation, advices and immense knowledge you will remain an unforgettable symbol and a great mentor for my PhD studies.

In addition, I would like to thank Prof. Dr. Reinhard Wetzker as my second advisor for giving me the opportunity to join his research group, supporting me with new ideas and encouragement – you showed me the importance of details toward great developments in science. Beside my advisors, I would like to thank also my Thesis Committee members: Dr. Christian Schmeer and Prof. Dr. Michael Bauer, for the insightful comments, ideas and support.

I would like to thank also my colleagues from the Institute of Molecular Cell Biology here in Jena, for the stimulating discussions, helping me to handle new research methods and having pleasant moments together. Special thanks go to my lab colleague Guang-Ping Lang for giving me a hand in Immunohistochemistry and Histology experiments.

Mrs. Dorith Schmidt, Mrs. Annette Böhmer and Mrs. Rose-Marie Zimmer deserve special thanks for helping me a lot and making the life easy here in Jena.

Last but not the least, I would like to thank my family, especially my parents (Isuf – father and Kumrije – mother), sister Driada, brother and his family (Benjamin, Shkendije and little Dorian) and my beautiful lovely wife Esra for standing behind me all the time and supporting me spiritually throughout my life in general – you all represent my strongest motivation in life.

"Life is like riding a bicycle. To keep your balance, you must keep moving." - ***Albert Einstein***

"The two most important days in your life are the day you are born and the day you find out why." - ***Mark Twain***

Ehrenwörtliche Erklärung

Hiermit erkläre ich ehrenwörtlich im Zusammenhang mit der Beantragung der Eröffnung meines Promotionsverfahrens:

- dass mir die Promotionsordnung der Medizinischen Fakultät der Friedrich-Schiller-Universität bekannt ist,
- ich die Dissertation selbst angefertigt habe und alle von mir benutzten Hilfsmittel, persönlichen, Mitteilungen und Quellen in meiner Arbeit angegeben sind, mich folgende Personen:
  - MTA Rose-Marie Zimmer: Unterstützung bei der Hirnpräparation, Histologie und Immunhistochemie;
  - Dr. Caroline Schmidt (CMB, Jena): Unterstützung bei ROS-Messungen,
  - Dr. Christian Marx (FLI, Jena): Unterstützung bei der Durchführung der Seahorse Assay-Messungen,
  - MSc. Guang-Ping Lang: Unterstützung bei der Histologie, Immunhistochemie und Auswertung der Phagozytose-Messungen;
  - Prof. Dr. Reinhard Bauer, bei der Herstellung des Manuskripts, Diskussion, Interpretation der Ergebnisse und Redigieren der Dissertation

  unterstützt haben,

- die Hilfe eines Promotionsberaters nicht in Anspruch genommen wurde und dass Dritte weder unmittelbar noch mittelbar geldwerte Leistungen von mir für Arbeiten erhalten haben, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen,
- dass ich die Dissertation noch nicht als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung eingereicht habe, und
- dass ich die gleiche, eine in wesentlichen Teilen ähnliche oder eine andere Abhandlung nicht bei einer anderen Hochschule als Dissertation eingereicht habe.

Jena, den 23.10.2018 Unterschrift des Verfassers:

.....